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Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7851–7855, November 1987
Biochemistry

pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse

(gene therapy/nude mouse/liposome composition/phosphatidylethanolamine)

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Communicated by John D. Baldeschwieler, July 20, 1987 (received for review February 4, 1987)

ABSTRACT **A plasmid containing the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene under the control of a mammalian cAMP-regulated promoter was entrapped in H-2K^k antibody-coated liposomes composed of dioleoyl phosphatidylethanolamine, cholesterol, and oleic acid (pH-sensitive immunoliposomes). The entrapped or free DNA was injected intraperitoneally into immunodeficient (nude) BALB/c mice bearing ascites tumor generated by H-2K^k-positive RDM-4 lymphoma cells. About 20% of the injected immunoliposomes were taken up by the target RDM-4 cells. Uptake was much less when liposomes without antibody were used. The presence of the targeting antibody on liposomes also significantly decreased the nonspecific uptake of liposomes by the spleen. Significant CAT enzyme activity was detected in RDM-4 cells from mice treated with DNA entrapped in the pH-sensitive immunoliposomes. Furthermore, CAT expression in RDM-4 cells was under the control of cAMP, as only the cells from mice injected with 8-bromo-cAMP and 3-isobutyl-1-methylxanthine showed CAT activity. CAT activity in liver and spleen was much lower (by factors of 12 and 5, respectively) than in the RDM-4 cells, and the activities in these reticuloendothelial organs were not regulated by cAMP. CAT activity in RDM-4 cells from mice injected with DNA entrapped in pH-insensitive immunoliposomes (containing phosphatidylcholine in place of phosphatidylethanolamine) was approximately one-fourth that in RDM-4 cells from mice injected with pH-sensitive immunoliposomes, indicating the superior delivery efficiency of the pH-sensitive liposomes. These results are discussed in terms of the DNA-carrier potential of immunoliposomes in therapy of cancer and genetic diseases.**

Correction of genetic disorders by gene therapy is one of the developing areas in medicine (1). The exogenous normal gene that is introduced may replace or coexist with the defective gene and produce normal gene product. Successful therapy of a genetic disorder requires knowledge of the structure, function, and regulation of the gene to be introduced into the deficient cell, as well as an efficient and specific means of delivering the gene to the target cell. Despite its great potential in medicine and biotechnology, gene therapy has not yet been used widely, mainly due to the poor efficiency of DNA delivery. Current methods of delivery of new genetic information into cells *in vitro* (for review, see ref. 1) include cell fusion, chromosome-mediated insertion, microcell-mediated gene transfer, liposome DNA carriers, spheroplast fusion, DNA-mediated gene transfer, microinjection, infection with recombinant RNA viruses, and infection with recombinant DNA viruses. However, most of these techniques are not applicable for use in animals or humans because of low efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and lack of target specificity.

To improve the efficiency of delivery of biologically functional molecules, pH-sensitive liposomes have been developed in several laboratories including ours (2, 3). These liposomes release their contents into the cytoplasm of target cells after they fuse with the endosomal membrane (4). In previous studies, we used a water-soluble fluorescent dye, calcein, as a convenient marker for observing cytoplasmic delivery (5) and used the herpes simplex virus thymidine kinase gene as a selectable marker for gene transfer (6). The results indicated that the liposome contents were released into the cytoplasm and that the transferred DNA can be expressed with high efficiency in target cells.

To increase specific binding of liposomes to target cells, acylated monoclonal antibodies were incorporated into the lipid bilayer of the liposome (7). The present study is designed to test the DNA-delivery potential of the pH-sensitive immunoliposomes in an animal model. We used RDM-4 lymphoma cells (provided by M. F. Mescher, Division of Membrane Biology, Medical Biology Institute, La Jolla, CA) as the target in this model system. These cells, which express the mouse major histocompatibility antigen H2-K^k, were grown as ascites tumor in the immunodeficient nude mouse of the BALB/c background (which expresses no H2-K^k antigen). The *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene was used as a convenient marker for observing gene transfer. This gene was placed under the control of a promoter that contained the cAMP regulatory sequence to test whether the expression of the foreign gene in the target cells could be regulated by an external signal such as cAMP.

MATERIALS AND METHODS

Materials. Dioleoyl phosphatidylethanolamine ([Ole$_2$]PtdEtn) and dioleoyl phosphatidylcholine ([Ole$_2$]PtdCho) were purchased from Avanti Polar Lipids. Oleic acid, cholesterol, acetyl coenzyme A, *n*-octyl glucoside, 8-bromo-cAMP (8-Br-cAMP), and 3-isobutyl-1-methylxanthine (iBuMeXan) were obtained from Sigma. SM-2 beads were purchased from Bio-Rad. [*dichloroacetyl*-1,2-^{14}C]Chloramphenicol was purchased from New England Nuclear. Anti-H2-K^k antibody (mouse IgG2a) was isolated from ascites fluid generated by hybridoma 11-4.1 (7) and was purified by protein A-Sepharose affinity chromatography (7). It was radioiodinated with ^{125}I and acylated with *N*-hydroxysuccinimide ester of pal-

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Abbreviations: CAT, chloramphenicol acetyltransferase; [^{3}H]CE, hexadecyl [^{3}H]cholestanyl ether; [Ole$_2$]PtdEtn, dioleoyl phosphatidylethanolamine; [Ole$_2$]PtdCho, dioleoyl phosphatidylcholine; iBuMeXan, 3-isobutyl-1-methylxanthine.

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mitic acid as described (7). BALB/c nude mice were purchased from Life Sciences (St. Petersburg, FL).

Plasmid. Plasmid pBBO.6-CAT (4.6 kilobase pairs) was a gift of W. D. Wicks (Department of Biochemistry, University of Tennessee, Knoxville). It was constructed from the plasmid pAZ1009 (8) by replacing the promoter region with a 621-base-pair upstream sequence of the rat phospho*enol*pyruvate carboxykinase gene, which contains a cAMP regulatory sequence (9). Details of the plasmid construction will be published elsewhere. Plasmid DNA was prepared and purified by standard techniques (10).

Liposome Preparation. Methods used for the preparation of large unilamellar immunoliposomes were based on the procedure of Philippot *et al.* (11), with modifications. Lipid films of various compositions (total lipid 10 μmol) were formed under a nitrogen stream and suspended in 10 mM Hepes/1 mM EGTA/150 mM NaCl, pH 8. Hexadecyl [^{3}H]cholestanyl ether ([^{3}H]CE) was included in the lipid mixture to monitor the lipid (12). The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted to 8. Palmitoylated anti-H2-K^k (1/25th of total lipid by weight), octyl glucoside (100 μmol), and DNA (150 μg) were added, and the mixture (final volume, 0.34 ml) was dialyzed at 4°C against 100 ml of 10 mM Tris·HCl/1 mM EDTA/150 mM NaCl (pH 8) and 1 g of washed SM-2 beads (13) overnight without stirring the dialysis buffer. Stirring was started the next morning. After 24 hr in dialysis, the buffer and beads were replaced with fresh ones and the dialysis was continued for an additional 24 hr. The liposomes were extruded through a polycarbonate filter of 0.2-μm pore diameter (Nuclepore) to obtain liposomes of uniform size distribution. Subsequently, the liposomes were separated from free DNA by gel filtration using a column of autoclaved Sepharose CL-2B (Pharmacia).

Inoculation of Mice. RDM-4 lymphoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.01% sodium pyruvate. RDM-4 cells (10^7) in 0.5 ml of phosphate-buffered isotonic saline (pH 7.4) were inoculated intraperitoneally (i.p.) in 6-week-old nude mice. After 7 days, the mice were injected i.p. with liposome-entrapped or free DNA at a dose of 20 μg of DNA (≈8.9 μmol of lipid) per mouse. Twenty-four hours later, 8-Br-cAMP (1 mg/100 g of body weight) and iBuMeXan (0.5 mg/100 g) were injected i.p. After 5 hr, ascites fluid was harvested from the peritoneal cavity by five 2-ml injections of phosphate-buffered saline. The ascites fluid, which contained RDM-4 cells and macrophages, was incubated in a glass Petri dish for 6 hr. The nonadherent RDM-4 cells were harvested from the supernatant, and adherent macrophages were harvested from the glass with a cell scraper. After centrifugation, cell pellets were resuspended in 0.25 mM Tris·HCl (pH 7.4) for CAT activity assay. The blood, hearts, spleens, lungs, stomachs, kidneys, and livers of the mice were also collected and homogenized. An aliquot of each homogenized organ was dissolved in Protosol (New England Nuclear) for measurement of ^{3}H by liquid scintillation counting. After centrifugation, the organ extracts were assayed for CAT activity. Two mice per group were used. Variations of data between experiments were less than 15%.

CAT Assay. CAT activity was determined by a modification of the method of Gorman *et al.* (14). Ascites cells were suspended in 125 μl of 0.25 mM Tris·HCl (pH 7.4) and frozen at −20°C until assayed. The cells were thawed, homogenized by sonication, and centrifuged. Protein concentration in the supernatant was measured by the method of Lowry *et al.* (15). [*dichloroacetyl*-1,2-^{14}C]Chloramphenicol (0.5 μCi; 1 μCi = 37 kBq) and 20 μl of 4 mM acetyl coenzyme A were added per 125 μl of cell or organ extract (3 mg of protein). The reaction was allowed to proceed at 37°C for 90 min. Chloramphenicol and its acetylated derivatives were extracted with ethyl acetate, separated by silica gel thin-layer chromatography, and visualized by autoradiography. Regions of the chromatogram containing the acetylated or nonacetylated chloramphenicol were then scraped from the plates and ^{14}C cpm were determined by liquid scintillation counting.

RESULTS

Characteristics of Liposomes. Two different lipid compositions were used for liposome preparation. Liposomes composed of [Ole$_2$]PtdEtn, cholesterol, and oleic acid (4:4:2 molar ratio) are pH-sensitive and become destabilized at pH below 6.0 (5). These liposomes were unilamellar and 0.29 ± 0.08 μm in diameter as determined by negative-stain electron microscopy. They entrapped 16.5% of the input DNA and contained ≈106 antibody molecules per liposome. Liposomes composed of [Ole$_2$]PtdCho, cholesterol, and oleic acid (4:4:2 molar ratio) are pH-insensitive (5). They were also unilamellar, were 0.18 ± 0.06 μm in diameter, contained ≈48 antibody molecules per liposome, and entrapped 14.4% of the input DNA. These two types of liposome, both filter-extruded, although different in size and pH-sensitivity, had the same antibody/lipid ratio.

Distribution of Liposomes in BALB/c Nude Mice. The distribution of liposomes was monitored by the radioactivity of [^{3}H]CE in several organs. This radiolabeled lipid marker has been shown to be a faithful marker for liposomes, and it is neither exchangeable with cellular lipids nor metabolizable by cells (12). Table 1 shows the distribution of liposomes in the mice. The recovery of [^{3}H]CE in these organs and cells was in the range of 60–70% of input ^{3}H cpm. Five different treatments were performed: (*i*) mice bearing RDM-4 cells were injected with pH-insensitive immunoliposomes that had been extruded through a 0.2-μm filter, (*ii*) mice bearing RDM-4 cells were injected with unextruded pH-sensitive immunoliposomes, (*iii*) mice bearing RDM-4 cells were injected with extruded pH-sensitive immunoliposomes, (*iv*) mice not bearing RDM-4 cells were injected with extruded pH-sensitive immunoliposomes, and (*v*) mice bearing RDM-4 cells were injected with antibody-free, pH-sensitive liposomes.

Distributions of radioactivity in spleens were markedly different in different groups. The non-tumor-bearing mice injected with immunoliposomes and the mice bearing RDM-4 cells but injected with antibody-free liposomes showed a very high degree of liposome accumulation in the spleen. In mice bearing RDM-4 cells and injected with immunoliposomes, the spleen accumulation was decreased by a factor of 3–4, no matter if the immunoliposomes were pH-sensitive or not, or whether they were extruded or not. Concomitantly, there was a 3- to 5-fold increase of liposome accumulation in the heart. Liposome uptake in nonadherent cells, the majority of which were RDM-4 cells, increased about 2-fold (≈20% of injected dose) when compared with uptake by nonadherent cells from mice treated with antibody-free, pH-sensitive liposomes. There was no significant change of ^{3}H distribution in livers, lungs, stomachs, kidneys, blood, and adherent cells of the ascites fluid.

Expression of CAT Gene in Ascites Cells. CAT gene expression was monitored by CAT enzyme activity, which is not found in eukaryotic cells. CAT activity was found in the RDM-4 cells (nonadherent cells in ascites fluid) of the mice injected with pH-sensitive immunoliposomes; however, the expression of the activity was dependent on cAMP stimulation (8-Br-cAMP plus iBuMeXan) and on the presence of antibody in the liposomes (Fig. 1). CAT activity was not found in extracts of the nonadherent cells without stimulation by 8-Br-cAMP plus iBuMeXan. CAT activity (443 pmol per mg per hr) in the nonadherent cells of the mice injected with pH-sensitive immunoliposomes was much higher than that

Table 1. Distribution of liposomes in BALB/c nude mice

Compartment	Distribution, %*				
	PIIL, ext.	PSIL, unext.	PSIL, ext.	PSIL, ext. (−tumor)†	PSL, ext.
Organs					
Liver	3.6 ± 0.0 (4.5 ± 0.0)	2.5 ± 0.3 (2.5 ± 0.5)	1.6 ± 0.7 (2.1 ± 0.9)	4.5 ± 1.4 (4.5 ± 0.4)	2.2 ± 0.9 (1.5 ± 0.3)
Heart	33.0 ± 1.9 (3.1 ± 0.3)	40.0 ± 7.9 (4.1 ± 0.2)	30.7 ± 12.2 (3.3 ± 1.3)	7.4 ± 2.7 (0.8 ± 0.1)	11.5 ± 3.2 (1.5 ± 0.7)
Spleen	40.0 ± 5.9 (8.1 ± 0.2)	43.8 ± 7.6 (4.0 ± 0.2)	30.5 ± 4.2 (5.1 ± 1.5)	145.0 ± 27.4 (22.2 ± 3.8)	144.4 ± 24.8 (29.5 ± 4.5)
Lung	13.5 ± 0.9 (1.5 ± 0.3)	8.8 ± 1.1 (1.0 ± 0.1)	13.0 ± 2.8 (2.0 ± 0.8)	6.5 ± 1.3 (0.5 ± 0.2)	6.2 ± 1.7 (1.1 ± 0.6)
Stomach	13.0 ± 3.1 (11.0 ± 4.4)	17.8 ± 2.3 (13.4 ± 6.8)	24.4 ± 3.3 (14.8 ± 6.5)	20.7 ± 1.3 (6.4 ± 1.9)	12.1 ± 4.2 (9.8 ± 6.6)
Kidney	3.9 ± 1.1 (1.3 ± 0.0)	9.6 ± 1.3 (2.5 ± 0.7)	7.0 ± 0.7 (2.4 ± 0.4)	2.1 ± 1.1 (1.1 ± 0.5)	3.0 ± 0.7 (1.5 ± 0.9)
Blood	1.0 ± 0.7 (1.2 ± 0.3)	0.7 ± 0.3 (0.1 ± 0.0)	0.6 ± 0.1 (0.1 ± 0.0)	0.6 ± 0.2 (0.1 ± 0.0)	0.4 ± 0.0 (0.2 ± 0.1)
Ascites cells					
Adherent	45.0 ± 3.9 (13.3 ± 0.6)	35.7 ± 11.4 (9.8 ± 4.2)	33.8 ± 7.5 (9.6 ± 1.9)	45.0 ± 3.9 (14.4 ± 0.9)	35.5 ± 4.5 (10.5 ± 0.2)
Nonadherent	49.5 ± 11.0 (16.6 ± 2.3)	37.1 ± 12.3 (18.9 ± 2.5)	57.3 ± 14.1 (20.5 ± 3.1)	—	24.5 ± 2.9 (10.1 ± 0.8)

PIIL, pH-insensitive immunoliposomes; PSIL, pH-sensitive immunoliposomes; PSL, pH-sensitive liposomes (no antibody); ext., extruded through 0.2-μm filter; unext., unextruded.
*Data (mean ± variation of two mice) are expressed as % of liposomes recovered per g of organ weight, per ml of blood, or per mg of ascites-cell protein; numbers in parentheses are the % of total injected liposomes.
†PSIL were injected into mice not carrying RDM-4 lymphoma cells.

(73 pmol per mg per hr) in the nonadherent cells of the mice injected with antibody-free liposomes (Table 2). Mice injected with free DNA showed no CAT activity in the cells. In this experiment, there was no detectable CAT activity in the adherent cells (containing macrophages) no matter what type of liposome was used. RDM-4 cells grown in tissue culture and not treated with liposomes also showed no activity.

The expression of CAT activity in the ascites cells seemed dependent on the type of liposomes used. Table 3 shows the data of an experiment in which three different kinds of liposomes were used. It is clear that filter-extruded pH-sensitive immunoliposomes were about 4 times more effective than filter-extruded pH-insensitive immunoliposomes for gene expression in the nonadherent cells (RDM-4 cells).

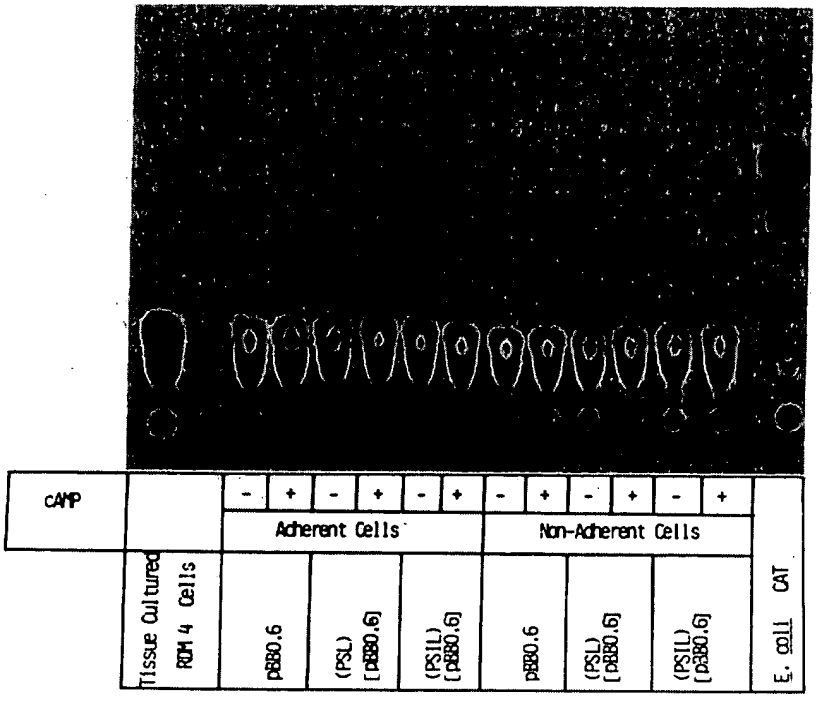

FIG. 1. CAT activity in ascites cells. High-mobility spots are acetylated [^{14}C]chloramphenicols. Low-mobility spot is the unmodified [^{14}C]chloramphenicol. [pBBO.6] stands for entrapped DNA, and pBBO.6 stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes. Both liposomes and immunoliposomes had been extruded through 0.2-μm filters.

Table 2. CAT activity in ascites cells and organs of nude mice

pBBO.6-CAT DNA	"cAMP" injection†	CAT activity*							
		Nonadherent cells	Adherent cells	Kidney	Liver	Heart	Spleen	Lung	Stomach
In PSIL	+	443	0	0	35	0	80	0	216
	−	0	0	4	73	0	86	0	134
In PSL	+	73	0	9	0	0	0	0	0
	−	0	0	0	0	0	19	0	120
Free	+	0	0	0	0	0	0	0	90
	−	0	0	0	0	0	0	0	60

PSIL, pH-sensitive immunoliposomes; PSL, pH-sensitive liposomes. Both PSIL and PSL had been extruded through 0.2-μm filters.
*Expressed as pmol of acetyl groups transferred per mg of protein per hr.
†Injection with 8-Br-cAMP and iBuMeXan.

Immunoliposomes that had not been filter-extruded, and thus were larger in size, seemed more efficient than those that had been extruded. In all cases, gene expression in the adherent cells (macrophages) was fairly low.

CAT Activity in the Organs of Nude Mice. To examine whether the foreign gene was expressed in the organs of nude mice, CAT activity in the organ extracts was also assayed. Extracts from kidney, heart, and lung showed no detectable CAT activity with any type of liposome treatment (Table 2). However, spleens and livers of the tumor-bearing mice treated with pH-sensitive immunoliposomes, but not with pH-insensitive immunoliposomes or free DNA, showed low levels of CAT activity. Extracts from stomachs of the treated mice showed variable amounts of CAT activity. A separate examination of untreated normal nude mouse showed similar CAT activity in the stomach. Since the food residues in the stomach were not removed before the extract was prepared, the CAT activity in the stomach extract was probably due to the microorganisms in the food residues.

DISCUSSION

This paper provides evidence that pH-sensitive immunoliposomes can mediate target-specific DNA delivery in an animal model. In the current study, the mice bearing no tumor cells but treated with immunoliposomes and the tumor-bearing mice treated with antibody-free liposomes showed a high degree of accumulation of liposome in the spleen, whereas the tumor-bearing mice treated with immunoliposomes had a much lower liposome accumulation in the spleen. Although the accumulation in the RDM-4 cells was only modestly (about 2-fold) increased in the latter case, it is clear that the biodistribution of the immunoliposomes is significantly different from that of the antibody-free liposomes. Nonspecific uptake by spleen cells is likely due to the reticuloendothelial cells (macrophages), which are also abundant in liver. However, accumulation in liver was fairly low. This observation may be unique to the nude mice since i.p. injected liposomes normally accumulate in the liver of mouse (16). Further studies are required to clarify this point.

Although the target RDM-4 cells were not the only cells in the mouse to which pH-sensitive immunoliposomes bound, the RDM-4 cells were the only ones that expressed significant CAT activity in response to 8-Br-cAMP plus iBuMeXan. The adherent cells (macrophages) isolated from the same peritoneal cavity did not express significant amounts of enzyme activity despite the fact that they accumulated some liposomes. Furthermore, the spleen cells in the non-tumor-bearing mice, which took up a large amount of the pH-sensitive immunoliposomes, showed only a very low level of gene expression. There are several possible explanations for this observation. It is possible that the liposomes were unstable in the mouse peritoneal cavity, resulting in separation of DNA from liposomes. The liposome remnants might have then entered the circulation via the thoracic duct and been taken up by the macrophages in the spleen. Other possibilities are that DNA was degraded in the macrophages or that the intact DNA was delivered but could not be efficiently expressed in macrophages because of the lack of cAMP regulatory factor(s). Wynshaw-Boris *et al.* (9) reported that such factor(s) may be tissue-specific. When liposomes containing [^{125}I]iodotyraminyl-inulin were intravenously injected into mice, the [Ole$_2$]PtdEtn/oleic acid liposomes were very leaky in the presence of plasma (17). However, the presence of cholesterol significantly increased the stability of [Ole$_2$]PtdEtn/oleic acid-containing liposomes (B. E. Tsusaki and L.H., unpublished observation). When [^{14}C]inulin was entrapped in ^{3}H-labeled liposomes composed of [Ole$_2$]PtdEtn, cholesterol, and oleic acid (4:4:2 molar ratio) and injected i.p. into nude mice, its distribution profile was similar to that of [^{3}H]CE (data not shown). Thus, cholesterol-containing pH-sensitive liposomes appear to be stable *in vivo*. This result suggests that intact liposomes were delivered to the macrophages, but that the DNA was either degraded and/or not expressed. Further studies are required to distinguish these possibilities.

Data from the CAT assays (Table 3) indicated that replacing the [Ole$_2$]PtdEtn of pH-sensitive immunoliposomes with [Ole$_2$]PtdCho to generate the pH-insensitive immunoliposomes decreased the ability of immunoliposomes to deliver DNA to RDM-4 cells. These results indicate that the pH-sensitive immunoliposome can release its contents into the cytoplasm of a target cell after it is endocytosed, whereas the pH-insensitive immunoliposome primarily delivers its contents to the lysosomes, where they are degraded. This is in agreement with previous conclusions (5, 18). The same experiment also showed that larger, unextruded liposomes were more effective delivery vehicles than the smaller, extruded ones (Table 3). This result is not consistent with our previous results of *in vitro* experiments in which extruded liposomes were more efficient in delivering the herpes simplex virus thymidine kinase gene to mouse Ltk$^-$ cells (6). Whether this discrepancy is due to a difference in cell types or growth conditions (*in vitro* vs. *in vivo*) is not known.

Nicolau *et al.* (19) reported gene expression in the livers of rats that received intravenous injection of the preproinsulin

Table 3. CAT activity in ascites cells of BALB/c nude mice

Liposome	CAT activity	
	Nonadherent cells	Adherent cells
PSIL, extruded	502	88
PSIL, unextruded	3119	114
PIIL, extruded	122	220

All mice were injected with 8-Br-cAMP plus iBuMeXan. PSIL, pH-sensitive immunoliposomes; PIIL, pH-insensitive immunoliposomes; extruded, liposomes were extruded through a 0.2-μm filter.

I gene encapsulated in antibody-free, conventional, pH-insensitive liposomes. This work clearly demonstrated the gene-delivery potential of liposomes *in vivo*. Nicolau's group (20) also showed that liposomes bearing galactosyl groups were targeted to hepatocytes. However, our work differs from their work in several important ways. Our previous work (6) and the work described here indicate that pH-sensitive immunoliposomes give more efficient cytoplasmic delivery than pH-insensitive immunoliposomes. The current study utilized an antibody instead of galactosyl groups to direct the liposomes to the target cells. Although the uptake of liposomes by reticuloendothelial cell-rich tissues such as spleen could not be avoided, our immunoliposome system exhibited target specificity in gene delivery.

The implications of our results for cancer therapy cannot be overlooked. DNA coding for cytotoxins such as diphtheria toxin (21) could be delivered to the target tumor cells. This type of chemotherapy is potentially superior to the immunoliposome-mediated delivery of conventional cytotoxic drugs such as methotrexate in several ways. First, the action of a gene should be much more potent than the action of a drug, due to a large degree of amplification through transcription and translation. Second, if DNA leaks out of the liposomes it should not be toxic because of rapid digestion by extracellular nuclease, whereas free cytotoxic drugs are toxic to normal tissues. Third, our results indicate that although the host organs took up liposomes, they did not significantly express the exogenous gene. This added level of specificity in cancer therapy may be very important, because by selecting appropriate control mechanisms, the delivered toxic gene may be expressed only in the tumor cells.

Our work also has implications for gene therapy. Two potential advantages of the antibody-coated liposomes in delivering DNA are their high transfer efficiency and safety. Successful human gene therapy will require efficient gene transfer and proper expression of the delivered gene in appropriate target cells (22). Retroviral vectors have been used as successful gene carriers (for review, see ref. 23). In many cases, the transferred genes were expressed in several hematopoietic cell types *in vitro* and in murine bone marrow stem cells. Although the retrovirus system has a much higher transformation efficiency than the conventional liposomes do, the viral oncogene and the random insertion of the retroviral genome into the host genome are undesirable side effects. The pH-sensitive immunoliposomes may be a good compromise in this regard. Furthermore, the fact that expression of the liposome-delivered gene can be properly controlled by an external signal, such as cAMP in the present study, is also an important advantage in human gene therapy.

We thank W. D. Wicks and his colleagues for the plasmid and for technical help. This work was supported in part by National Institutes of Health Grant CA24553 and a contract from the Martin Marietta Corporation.

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secreted by transfected COS or Chinese hamster ovary cells begins at a glycine residue at position 21; the non-glycosylated form has a molecular mass of 12,397 by electrospray mass spectrometry, close to the predicted M_r of 12,399, although it migrates as a 9K protein on SDS-PAGE (Fig. 1*C*).

The production by monocytes of inflammatory cytokines such as IL-1, IL-6 and tumour necrosis factor-α (TNF-α) is a crucial initiating event in a number of infectious and inflammatory pathologies. We have found that IL-13 strongly inhibits IL-6 secretion induced by bacterial lipopolysaccharide (LPS) in peripheral blood mononuclear cells (PBMC) (Fig. 3*a*). IL-13 would appear to be acting directly on monocytes because an inhibition of IL-6 mRNA accumulation is observed rapidly (within 4 hours) in cultures of PBMC enriched in monocytes by adherence to tissue culture dishes (Fig. 3*b*). A marked inhibition is also seen for other inflammatory monokine mRNAs (IL-1β, TNF-α, IL-8, gro-β) in LPS-treated monocytes in the presence of IL-13 (Fig. 3*b*, and other data not shown). The action of IL-13 would thus seem to be a generalized block on inflammatory monokine synthesis, a property shared with the other Th2 lymphokines IL-4 and IL-10 (ref. 13). IL-13 and IL-4 show similar levels of inhibition of IL-6 synthesis (Fig. 3*a*). IL-13 also inhibits production of human immunodeficiency virus (HIV) by tissue-culture differentiated macrophages[14], contrasting with the stimulatory effects of macrophage-activating cytokines such as IL-3 and GM-CSF on HIV production that have been reported in comparable conditions[15].

The production of IFN-γ by large granular lymphocytes (LGL) may direct subsequent immune responses, leading to macrophage activation and to a 'Th1-type' cellular immune response[16]. The major cytokine influencing this production of IFN-γ is IL-2 (ref. 17). We have found that IL-13 has a small, direct effect on IFN-γ synthesis by LGL, and synergizes with both suboptimal and optimal doses of IL-2 (Fig. 3*c*). In this respect it resembles IL-12 (ref. 17), rather than IL-4, which strongly inhibits IFN-γ synthesis by these cells (Fig. 3*c*).

IL-13 also affects B lymphocytes, increasing their proliferation and the expression of the CD23 surface antigen (P. Carayon and T. Defrance, personal communication). IL-13 is thus a highly pleiotropic cytokine. In its anti-inflammatory effects on monocytes and its stimulation of the humoral response through B lymphocytes, IL-13 contributes to the 'Th2-type' response together with IL-4 and IL-10 (refs 18, 19). In, however, its effects on IFN-γ synthesis, it might be expected to promote a 'Th1-type' cellular immune response[16,20]. A full understanding of the cytokine network in different pathological situations now needs to take into account the activities of IL-13.

The anti-inflammatory function of IL-13 may be crucial in clinical inflammation, for example in septic shock[21] or rheumatoid arthritis[22]. Its activity on LGL may be clinically interesting in that, unlike IL-4, it does not decrease and can even increase the IL-2-induced lymphokine-activated killer activity of these cells (our unpublished results). As IL-13 also inhibits HIV replication *in vitro*[14], and systemic immunity to parental tumour cells can be induced by IL-13-secreting tumour cells *in vivo* (D. Fradelizi, personal communication), IL-13 would appear to represent a potentially important new member of the therapeutic cytokine arsenal. □

Received 15 December 1992; accepted 25 February 1993.

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ACKNOWLEDGEMENTS. We thank B. and M. Delpech, M. Goncalves and M. F. Jamme for technical assistance, P. Carayon, M. Magazin, B. Pessegue, and N. Vita for discussion, and P. Ramond for help and advice on DNA sequence comparison.

Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy

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CYSTIC fibrosis (CF) is a lethal inherited disorder affecting about 1 in 2,000 Caucasians. The major cause of morbidity is permanent lung damage resulting from ion transport abnormalities in airway epithelia that lead to mucus accumulation and bacterial colonization. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene[1] that encodes a cyclic-AMP-regulated chloride channel[2,3]. Cyclic-AMP-regulated chloride conductances are altered in airway epithelia from CF patients[4-6], suggesting that the functional expression of *CFTR* in the airways of CF patients may be a strategy for treatment. Transgenic mice[7-9] with a disrupted *cftr* gene are appropriate for testing gene therapy protocols. Here we report the use of liposomes to deliver a *CFTR* expression plasmid to epithelia of the airway and to alveoli deep in the lung, leading to the correction of the ion conductance defects found in the trachea of transgenic (*cf/cf*) mice. These studies illustrate the feasibility of gene therapy for the pulmonary aspects of CF in humans.

Plasmid DNA complexed with cationic liposomes can be successfully delivered and expressed in airway epithelia of rodents[10,11]. A suitable plasmid for expressing CFTR protein was constructed in the vector pREP8 (see legend to Fig. 1). In this plasmid, pREP8-CFTR, the human *CFTR* complementary DNA is under transcriptional control of the constitutive Rous sarcoma virus (RSV) 3' long terminal repeat (LTR) promoter, known to be active in nonproliferating airway epithelial cells[10]. To show that pREP8-CFTR expresses CFTR protein after

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transfection, plasmid DNA complexed with cationic liposomes was introduced into HeLa cells and CFTR protein detected by western blotting (Fig. 1*a*). To ascertain whether the expressed CFTR protein was functional, cAMP-stimulated iodide efflux was measured in transfected cells (Fig. 1*b*). In HeLa cells transfected with pREP8-CFTR, iodide efflux was stimulated by a cAMP-agonist cocktail. The cocktail did not stimulate efflux from cells transfected with the vector pREP8. The characteristics of this cAMP-stimulated anion efflux were similar to those reported previously for CFTR-expressing cells[12,13]. Thus, cells transfected with pREP8-CFTR express functional CFTR protein.

RNA *in situ* hybridization was used to demonstrate that the CFTR expression plasmid can be delivered to airway epithelial cells by liposome-mediated transfection *in vivo*. Because *CFTR* messenger RNA is expressed at high levels in human and rodent intestinal crypts[14-16], mouse intestinal sections were used as controls to demonstrate probe specificity. No hybridization to mouse intestine was detected with either of two human *CFTR* probes whereas, in consecutive sections, the mouse antisense *cftr* probe (but not the sense probe) detected abundant *cftr* mRNA in the crypts (data not shown). Additionally, neither the antisense nor the sense *hisD* vector control probes hybridized with mouse intestinal mRNA, as expected (data not shown). This demonstrates that the human *CFTR* and vector *hisD* probes do not cross-hybridize with mouse *cftr* mRNA.

After transfection of pREP8-CFTR DNA into the airways of mice of 20–28 days old, sequences corresponding to human *CFTR* were detected by *in situ* hybridization (Fig. 2). Strong hybridization signals were observed in isolated groups of airway cells using both the human *CFTR* probe (Fig. 2*a–c*) and the *hisD* vector-specific probe (*d–f*). In a series of consecutive sections the hybridization signals observed with the human *CFTR* and *hisD* probes colocalized to the same airways and airspaces (Fig. 2*a–f*). No hybridization was detected with the mouse *cftr* probe. This provides strong evidence that the hybridization signals obtained are highly specific and due to the transfected plasmid. Hybridization of these same probes to lung sections from untransfected animals served as a negative control against nonspecific hybridization; neither the human *CFTR* probe (*g–i*) nor the *hisD* probe (*j–l*) hybridized to any mRNAs in the lungs of untransfected mice. Hybridization signals were obtained with both the sense and antisense probes (Fig. 2*a–f*). Normally the antisense probe is used to detect mRNA whereas the sense probe serves as a negative control. Following transfection, however, both the sense and antisense probes would be expected to recognize vector DNA. The stronger signal observed with the antisense probe indicates transcription. This was seen for the *hisD* gene which is transcribed from a vector promoter. In most hybridizing cells, the signal obtained with the antisense human *CFTR* probe (Fig. 2*b*) was also greater than that obtained with the sense probe (*c*), implying that human *CFTR* mRNA is expressed following transfection.

The data in Fig. 3 show hybridization to sections through different regions of the lungs of a mouse which had been transfected with pREP8-CFTR. No expression of endogenous mouse *cftr* mRNA was detected in any region of the lung (data not shown), consistent with previous studies showing low-level *cftr* expression in rodent lung[14], and with detailed studies of these transgenic animals (A.E.O.T. *et al.*, manuscript in preparation). This shows that the transfection protocol does not induce expression of endogenous mouse *cftr* mRNA. Human *CFTR*

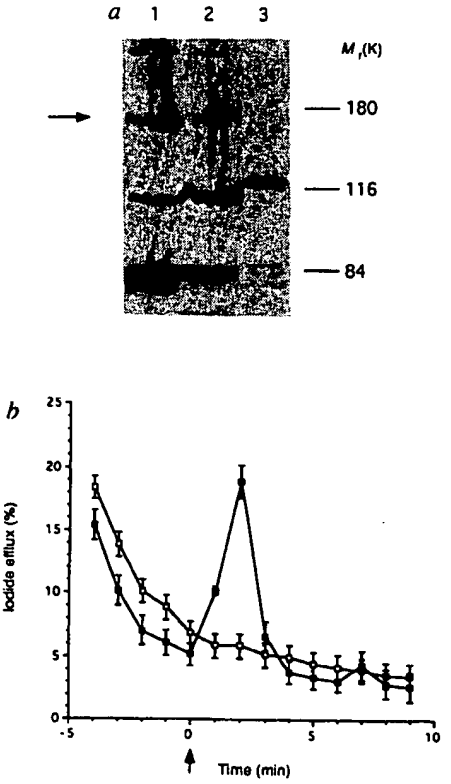

FIG. 1 Expression of functional CFTR protein from plasmid pREP8-CFTR in HeLa cells. *a*, Western blot confirming expression of *CFTR* after transfection of HeLa cells using Lipofectin. Lanes 1, HT29 cells; 2, HeLa cells transfected with pREP8-CFTR; 3, HeLa cells transfected with the vector pREP8. The HT29 cells served as a positive control for *CFTR* expression and migration[25,26], indicated by the arrow. The ~115 and 85K bands are due to nonspecific cross reactions of the antibody[25]. M_r ×1,000 of markers is indicated. *b*, Time course of iodide efflux from HeLa cells. Cells were transfected with plasmid pREP8-CFTR (■) or the vector pREP8 (□). The arrow indicates the point at which a cAMP-agonist cocktail was added. The data are displayed as the mean of three individual experiments (±s.e.m.), expressed as a percentage of the total efflux.

METHODS. Human *CFTR* cDNA encoding the entire *CFTR* coding sequence[1] (nucleotides 133–4,620) was inserted into the plasmid pREP8 (Invitrogen), under transcriptional control of the RSV 3′ LTR promoter, to create plasmid pREP8-CFTR. The cDNA incorporated three minor changes from the published sequence (C to G at nucleotide 136[13]; T to C at nucleotide 936[27]; A to C at nucleotide 1990[28]), and included a Kozak translation initiation sequence[29] (CCACCATG) immediately 5′ to the translation initiation codon. For plasmid transfection, 1×10^6 HeLa cells were seeded into each well of 35-mm, 6-well tissue culture dishes in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, and incubated at 37 °C. After 24 h growth, cells in each well were transfected with 8 μg plasmid DNA mixed with 13 μg Lipofectin (Gibco BRL) and diluted to 3 ml in Optimem 1 (Gibco BRL). After a further 24–48-h incubation at 37 °C, cells were either collected for protein extraction or used for anion efflux measurements. For protein extraction, cells were washed five times with ice-cold PBS and collected into a buffer containing 10 mM Tris-Cl pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, and the protease inhibitors antipain (50 μg ml^{-1}), aprotinin (10 μg ml^{-1}), benzamidine (310 μg ml^{-1}), leupeptin (5 μg ml^{-1}), pepstatin A (5 μg ml^{-1}) and phenylmethylsulphonyl fluoride (175 μg ml^{-1}). Cells were lysed by repeated passage through a 19-gauge needle. Cellular and nuclear debris were removed from the lysate by a 5-min centrifugation at 300*g* and membranes pelletted by a 30-min centrifugation at 100,000*g*. The membrane pellet was dissolved in 2.5%Triton X-100 and separated by electrophoresis on a 6% SDS-polyacrylamide gel. CFTR was detected by western blotting after transfer to a Hybond C-super membrane (Amersham) using the well characterized anti-CFTR antisera 181[25]. Immunodetection was by enhanced chemiluminescence (ECL; Amersham). To measure cAMP-stimulated efflux, the transfected cells were preloaded with iodide by incubation for 40 min at room temperature in 3 ml loading buffer (136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, 20 mM HEPES, pH 7.4). Extracellular NaI was removed by 6 × 1 ml rinses in efflux buffer (loading buffer with 136 mM $NaNO_3$ replacing the NaI). Cells were then washed with 1 ml efflux buffer for 1 min using a sample-replace procedure. After the fifth 1-min sample (designated time 0), cAMP-agonists (1 mM 3-isobutyl-1-methylxanthine (IBMX), 200 μM dibutyryl-cAMP, 10 μM forskolin, dissolved in DMSO) were included in the efflux buffer. The concentration of iodide in each 1-ml aliquot was determined using an iodide-specific electrode (HNU systems).

expression was seen in the airways of three out of four animals transfected with pREP8-CFTR and, in at least one transfected animal, human *CFTR* sequences were detected in all five lobes of the lung. Positive cells were detected in large and small airways (Fig. 2*a–d*), and in cells lining the air spaces of the more distal regions of the lung (*e–j*). It appeared to be the surface epithelial cells of the airways that had been transfected. Colocalization of the *CFTR* signal with the *hisD* probe (Fig. 2), confirmed that the signal was a consequence of transfection. Thus, transfection is effective and expression of human *CFTR* throughout the airway was achieved.

To determine whether delivery of *CFTR* cDNA to the airways could correct the ion transport defects apparent in CF, we used a recently developed mouse model[9,17]. These transgenic (*cf/cf*) mice are homozygous for a null mutation in *cftr* and express little or no detectable endogenous *cftr* mRNA (A.E.O.T. *et al.*, manuscript in preparation). CFTR-dependent, cAMP-stimulated chloride conductances are greatly reduced in the airways

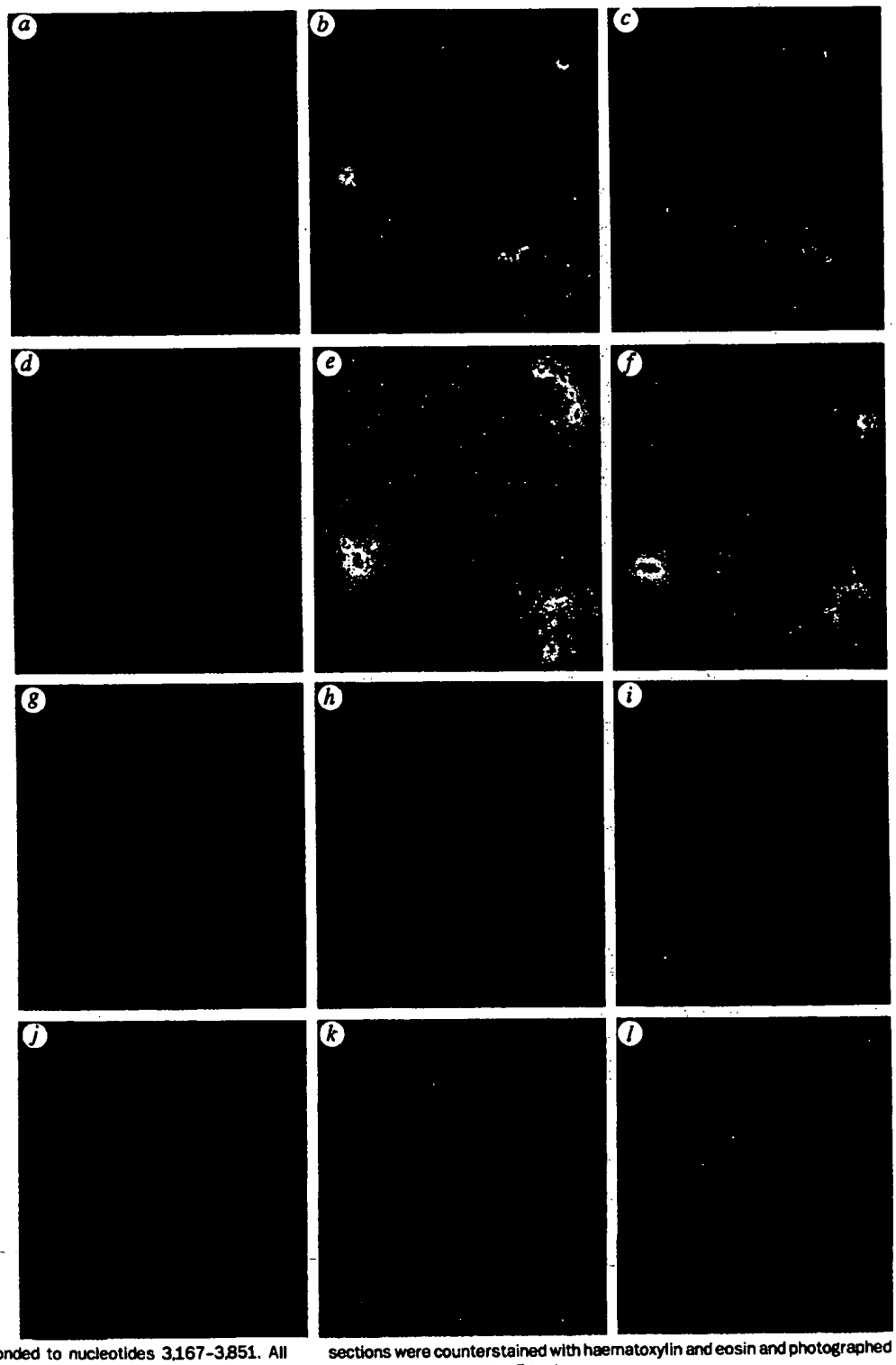

FIG. 2 Detection of human CFTR by *in situ* hybridization in mouse airways following *in vivo* transfection. *a–f*, Data obtained for a mouse transfected with pREP8-CFTR; *G–l*, controls for an untransfected mouse. The probes used were against human *CFTR* exons 1–6 (*a–c* and *g–l*) or the *hisD* vector sequences (*d–f* and *j–l*). For each example, three panels are shown: (1) a brightfield view of a section hybridized with the antisense probe, to illustrate tissue morphology (*a, d, g, j*); (2) a darkfield view of the same section (*b, e, h, k*); (3) a darkfield view of an adjacent section probed with the control sense probe (*c, f, i, l*). Scale bar, 200 μm. Similar results were obtained with several animals.

METHODS. Mice were given enough avertin by intraperitoneal injection to induce very light anaesthesia. For transfection, ~100 μg plasmid DNA was mixed with 25 μg Lipofectin in a total volume of 50 μl and administered to mice by tracheal instillation in two loads by insertion of a metal applicator, adapted from a 25-gauge blunted syringe needle, through the mouth and into the trachea to the point where the main bronchi branch off. The animals used weighed between 5 g and 12 g. Four days after transfection, *in situ* hybridization was performed on perfusion-fixed tissue by a modification of the method described by Simmons *et al.*[30], as described previously[15]. ^{35}S-labelled RNA probes were synthesized *in vitro* by run-off transcription from plasmid DNA, incorporating [^{35}S]UTP. The antisense and sense (control) probes were derived from opposite strands of the same plasmid. The plasmids used for probe generation were as follows. The two human *CFTR* probes, corresponding to nucleotides 62–645 (exons 1–6) and nucleotides 1,977–2,461 (exon 13) (numbering according to ref. 1), have been described previously[16]. The mouse cftr probes were derived by reverse transcriptase PCR from mouse testis mRNA and corresponded to nucleotides 305–691 of exons 3–5. The *hisD* vector probe was subcloned from pREP8 and corresponded to nucleotides 3,167–3,851. All probes were cloned into Bluescript vectors (Stratagene). After developing, sections were counterstained with haematoxylin and eosin and photographed using a Nikon Microphot FX microscope.

and caeca of these mice, compared with normal (+/+) animals, mimicking features of the human disorder[17]. The mice frequently die shortly after birth as a consequence of intestinal blockages[9]. Ion transport in the trachea was measured by voltage clamping at zero potential, using pharmacological agents to eliminate or stimulate various processes (Fig. 4). Measurements were also made with the caecum of the same animal as an internal control. Figure 4*A* shows a set of typical results, and Fig. 4*B* a compilation of the data. For each tracheal preparation, three measurements were made: amiloride-sensitive sodium absorption (labelled Na^+), cAMP-stimulated chloride secretion (labelled Cl^- cAMP), and Ca^{2+}-stimulated chloride secretion (labelled Cl^- Ca^{2+}). As expected, CFTR-dependent, cAMP-stimulated chloride secretion was significantly reduced ($P < 0.01$) in both the tracheas and caeca of the *cf/cf* mice compared with the normal (+/+) mice. There was no significant difference in the cAMP-stimulated chloride secretion between untreated and pREP8-transfected normal mice, indicating that transfection itself has no effect on ion transport. Most importantly, transfection of *cf/cf* mice with pREP8-CFTR restored the cAMP-stimulated chloride secretion in the trachea to a level comparable with that of normal (+/+) animals. In sharp contrast, transfection of the *vf/cf* mice with the vector pREP8 had no significant effect on the cAMP-stimulated chloride secretion in the trachea. The caecum of *cf/cf* mice transfected with pREP8-CFTR showed no appreciable cAMP-stimulated chloride secretion

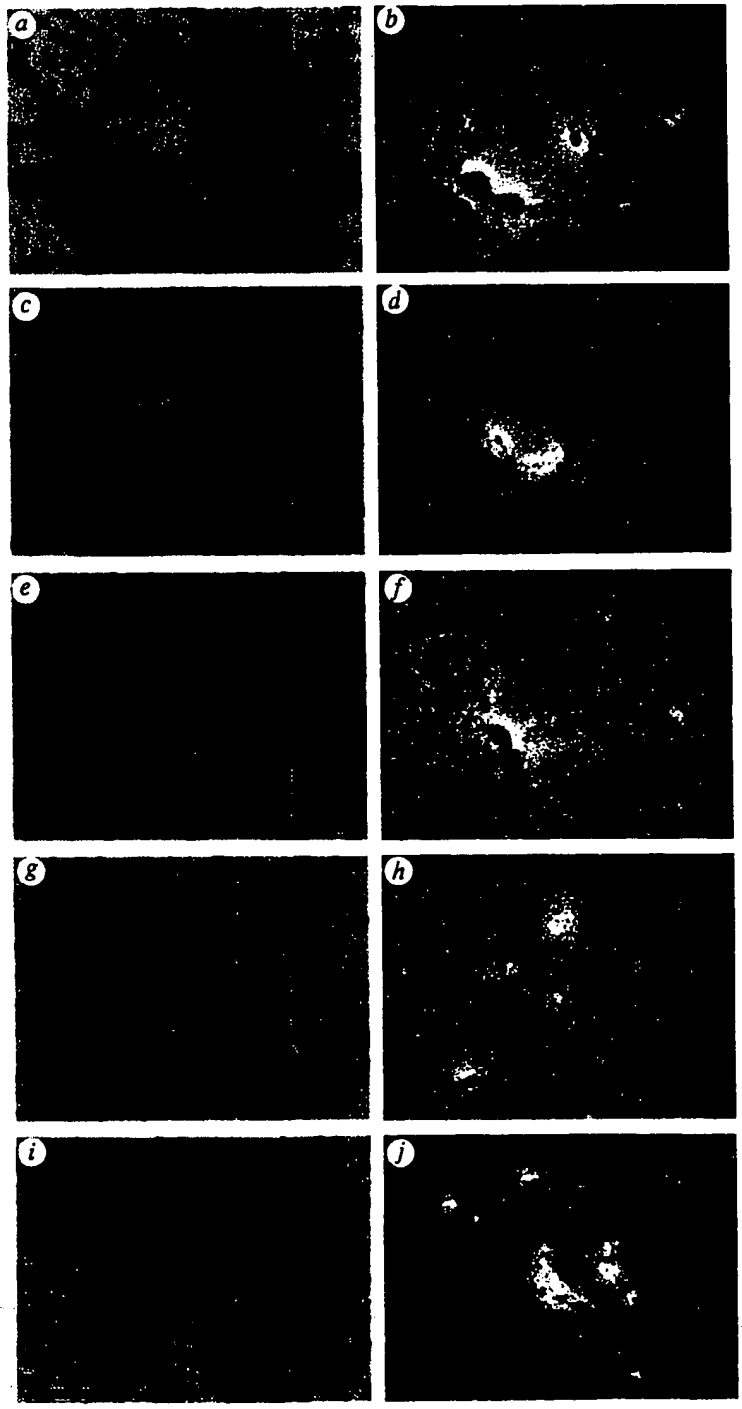

FIG. 3 Detection of human CFTR in different regions of the mouse airway following transfection. Sections from different regions of the airway of a mouse transfected with pREP8-CFTR were hybridized with human antisense *CFTR* probes corresponding to either exons 1–6 (*a–f, i, j*) or exon 13 (*g* and *h*). *a–d*, Expression of human *CFTR* in small and large airways; *e–j*, expression in airspaces in the more distal regions of the lung. Some variation was found in the proportion of cells transfected in different animals which probably reflects differences in the amounts of DNA delivered. Scale bar, 100 μm. See legend to Fig. 2 for details of methods and hybridization probes.

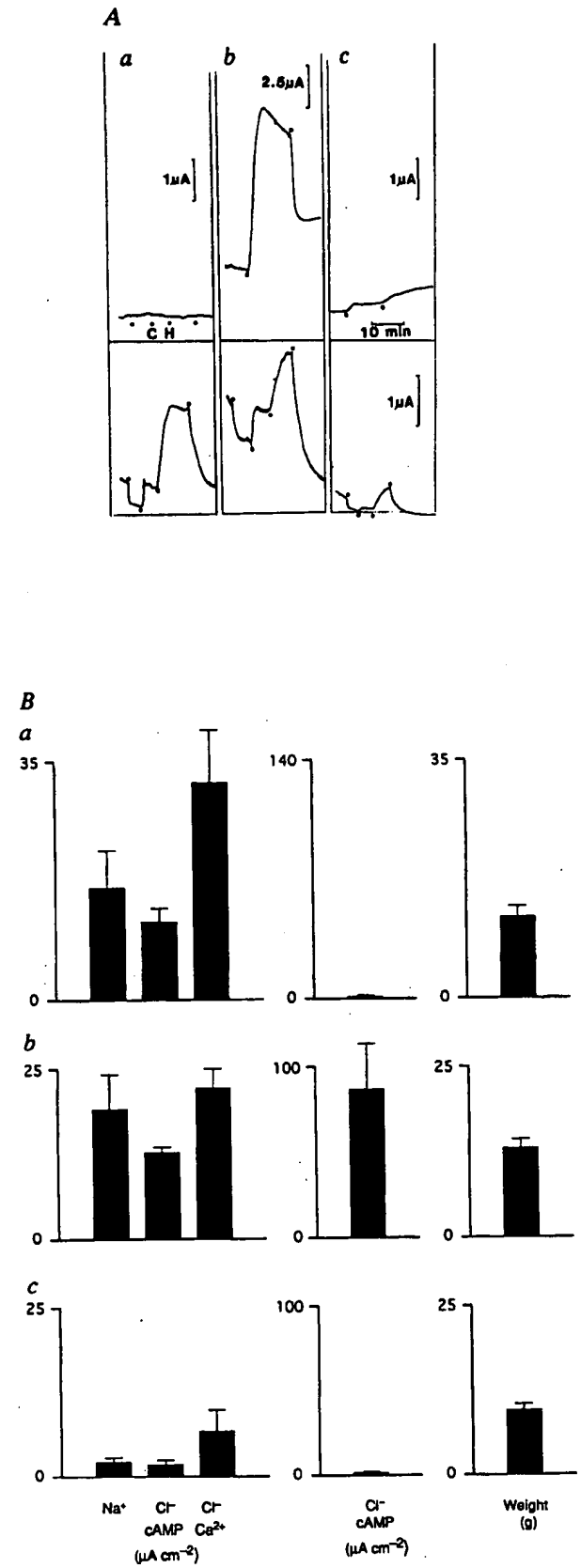

FIG. 4 Correction of the ion channel defects in the trachea of transgenic (*cf/cf*) mice. *A*, Sample traces showing examples of the data from which panel *B* was compiled. Three paired tracheal/caecal preparations are shown. Upper records show measurements for the caecum and lower records for the trachea of the same animal. *a*, *cf/cf* mouse transfected with pREP8-CFTR; *b*, +/+ mouse transfected with pREP8; *c*, *cf/cf* mouse transfected with the vector pREP8. Four additions were made to each of the tracheal preparations at the time points indicated by dots. First, amiloride (100 μM) was added (apically) to block electrogenic sodium absorption and to ensure subsequent current increases were not due to this activity. EC_{50} for amiloride is ~1 μM and 100 μM will give essentially 100% inhibition[31]. Second, forskolin (10 μM) was added to both sides of the membrane to stimulate adenylate cyclase and activate cAMP-sensitive chloride channels, increasing chloride secretion. Third, the Ca^{2+} ionophore A23187 (1 μM) was added to both sides of the membrane to activate Ca^{2+}-dependent chloride secretion. The ionophore-induced responses were much slower than those induced by forskolin. Finally, frusemide (1 mM) was added basolaterally to block chloride secretion, confirming the nature of the electrogenic transporting activity. Frusemide (1 mM) inhibits over 90% of Cl^- secretion[17]. Calibrations for the trachea are the same in each panel. Caecal preparations (upper records) received two additions, forskolin (10 μM, added to both sides of the membrane) and frusemide (1 mM, added basolaterally). Other additions are specifically labelled: C, carbachol (10 μM); H, histamine (10 μM). In both the caecum and the trachea the chloride secretory responses were inhibited by frusemide, indicating that they are due to electrogenic chloride secretion from the basolateral to the luminal side of the epithelium. In 8 out of 10 *cf/cf* caeca, frusemide led to a slight increase in short-circuit current (SSC) (*c*, upper record); this is probably due to a blockage of K^+ secretion and is typical of the caecum of *cf/cf* mice[17]. *B*, Compilation of the data. Mice were subjected to three different treatment protocols: *a*, *cf/cf* mice transfected with pREP8-CFTR; *b*, +/+ mice transfected with the vector pREP8; *c*, *cf/cf* mice transfected with the vector pREP8. Four animals in each group were matched based on a compromise between weight and age. Data were only included when paired airway and caecal measurements could be made for the same animal. The genotypes of the transfected mice, and of the plasmid DNA with which they were transfected, was unknown at the time the measurements were made. For each treatment regime, three sets of data are shown. (1) The left hand columns show SCC measurements for the trachea. Three measurements of SCC changes are presented: Na^+, amiloride-sensitive sodium absorption[31]; Cl^- cAMP, SCC change induced by forskolin, presumed to reflect CFTR function[17,32]; Cl^- Ca^{2+}, SCC change induced by the addition of the calcium ionophore A23187. As about 50% of the basal current in the airways was due to sodium absorption, chloride secretion was measured after the addition of amiloride (100 μM) which abolished electrogenic sodium absorption. (2) The central columns show SCC measurements for the caecum. Only cAMP-sensitive chloride secretion (Cl^- cAMP), induced by the addition of forskolin (10 μM), was measured. Amiloride was not added because the caecum shows no sodium absorptive current[17,33]. (3) The right-hand columns show the weights of the animals used (mean ± s.e.m.). Note: the ion transport characteristics of 4/6 pREP8-CFTR transfected *cf/cf* mice were altered by transfection; the reason for the failure of the other two mice is almost certainly failure in delivery. Nevertheless, the forskolin-sensitive SCC (Cl^- cAMP) in the whole group including the two failures (9.2 ± 2.6 μA cm^{-2}, $n=6$) was significantly greater ($P<0.05$, Mann and Witney test) than the value for *cf/cf* mice (1.9 ± 0.5 cm^{-2}, $n=4$). Finally, data for two other groups of animals were obtained although these are not illustrated in the figure. Untreated, wild-type (+/+) mice ($n=5$; weight ±s.e.m. = 32.2 ± 2.9 g) had transport parameters as follows (mean ± s.e.m.): for the trachea $Na^+ = 10.7 \pm 4.8$ μA cm^{-2}, Cl^- cAMP = 11.4 ± 4.3 μA cm^{-2}, Cl^- $Ca^{2+} = 12.1 \pm 4.7$ μA cm^{-2}; for the caecum Cl^- cAMP = 35.6 ± 7.0 μA cm^{-2}. Heterozygous (cf/+) mice transfected with pREP8-CFTR ($n=2$; mean weight, 7.0 g) had the following transport parameters (mean ± s.e.m.): for the trachea: $Na^+ = 4.5$ μA cm^{-2}, Cl^- cAMP = 6.6 μA cm^{-2}, Cl^- $Ca^{2+} = 8.2$ μA cm^{-2}; for the caecum Cl^-cAMP = 104.6 μA cm^{-2}. Note that the forskolin-sensitive currents (Cl^- cAMP) in the trachea were smaller than those reported previously for wild-type mice[17]. This is undoubtedly a consequence of edge damage caused by using only 2.27 mm^2 areas of trachea in the present study, necessitated by the small size of the *cf/cf* mice, compared with 4 mm^2 areas of trachea in previous studies.

METHODS. Transgenic mice were genotyped by PCR and/or Southern blot analysis as described[17]. Introduction of plasmid DNA into the mouse airways was as described in the legend to Fig. 2. Trachea and caeca were removed from the transfected animals killed by exposure to 100% CO_2. A single tracheal preparation (2.27 mm^2) and a single caecal preparation (20 mm^2) was prepared from each animal. The reduction in tracheal area, compared with a previous report[17] was due to the necessity of using animals as small as 5 g. The trachea were cleaned and cut longitudinally along the dorsal surface and a piece placed under microscopic control in a specially constructed Ussing chamber designed to preserve the curvature of the tissue. Electrogenic ion transport was measured directly as SCC recorded by voltage clamping the tissue at zero potential, as described previously[17].

compared with the control (+/+) mice, confirming the genotypes of the mice and that the transfection procedure did not affect the gut. Thus, the transfection procedure used can restore CFTR-dependent, cAMP-stimulated chloride secretion by airway epithelia to normal levels.

In the airways of human CF patients there is an increase in amiloride-sensitive sodium absorption, as well as a decrease in chloride secretion, compared with controls[18–20]. It has been suggested that this is crucial to the development of the disease state, as application of amiloride by aerosol alleviates the decline in lung function in CF[21,22]. It is not yet clear how a loss of CFTR function leads to this increase in sodium absorption. In contrast to the human, sodium absorption was reduced in the airways of *cf/cf* mice (Fig. 4*B*). Transfection of the *cf/cf* mice with pREP8-CFTR, but not with the vector pREP8, significantly increased sodium absorption (seven- to eightfold; $P < 0.05$), to essentially wild-type (+/+) levels (Fig. 4*B*). Thus, secondary alterations in sodium transport were also corrected to wild-type levels by the transfection protocol used. Finally, Ca^{2+}-induced chloride secretion reflects an alternative pathway for chloride secretion in the airways distinct from the CFTR pathway[4,23]. Ca^{2+}-stimulated chloride secretory currents were not defective in *cf/cf* trachea, compared with trachea of normal (+/+) mice, but were significantly increased following transfection with *CFTR* ($P < 0.05$; Fig. 4*B*). This latter increase is probably a consequence of hyperpolarization through Ca^{2+}-sensitive K^+ channels, which increases the electrochemical gradient for Cl^- exit through the introduced CFTR channels and the pre-existing second pathway[24].

These data show that the ion transport defects in CF can be corrected *in vivo*. Liposomes, which in clinical trials have been shown to be non-toxic and non-immunogenic, may be safer than viral vectors which have the inherent risks of immunogenicity, replication and transmission. Our results illustrate the invaluable role of transgenic null *cf/cf* mice in assessing the efficiency of various gene therapy approaches. We have shown that functional expression of CFTR not only corrects the primary ion transport defect of the trachea (that is, the cAMP-stimulated chloride secretion), but also corrects secondary alterations in sodium absorption which are a consequence of loss of CFTR function. There seems to be no reason why this approach should not be transferable to humans for the treatment of the pulmonary features of CF. □

Received 4 February; accepted 23 February 1993.

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ACKNOWLEDGEMENTS. We thank S. Tucker for advice on the use of the iodide electrode, C. Wardle for advice on western blotting and A. Harris for helpful discussions. A.E.O.T. is a Beit Memorial Fellow. C.F.H. is a Howard Hughes International Research Scholar. This work was funded by the Wellcome Trust, the Cystic Fibrosis Research Trust and the Imperial Cancer Research Fund.

Germ-line transmission and expression of a human-derived yeast artificial chromosome

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INTRODUCTION of DNA fragments, hundreds of kilobases in size, into mouse embryonic stem (ES) cells would greatly advance the ability to manipulate the mouse genome. Mice generated from such modified cells would permit investigation of the function and expression of very large or crudely mapped genes. Large DNA molecules cloned into yeast artificial chromosomes (YACs) are stable and genetically manipulable within yeast[1], suggesting yeast-cell fusion as an ideal method for transferring large DNA segments into mammalian cells. Introduction of YACs into different cell types by this technique has been reported[2–8]; however, the incorporation of yeast DNA along with the YAC has raised doubts as to whether ES cells, modified in this way, would be able to recolonize the mouse germ line[5]. Here we provide, to our knowledge, the first demonstration of germ-line transmission and expression of a large human DNA fragment, introduced into ES cells by fusion with yeast spheroplasts. Proper development was not impaired by the cointegration of a large portion of the yeast genome with the YAC.

Yeast spheroplasts, carrying yHPRT, a 670 kilobase (kb) YAC containing the human hypoxanthine phosphoribosyltransferase (HPRT) gene[4], were fused with the HPRT-deficient ES cell line E14TG2a (ref. 9). Clones expressing the HPRT locus were selected in hypoxanthine/aminopterin/thymidine (HAT) medium (Fig. 1 legend) and expanded. The human HPRT gene was detected by hybridization in all ES cell clones analysed (not shown). The integration of additional human sequences was examined by comparing the *Alu* profile of 37 HAT-resistant (ESY) clones to that of yHPRT in yeast. Most, if not all, of the 30 *Alu* fragments characteristic of yHPRT were present and of similar relative intensity in over 90% of the ESY clones (Figs 1*a*, 3*B*). In clones with an incomplete *Alu* profile (such as ESY 8-5, Fig. 1*a*) only a few fragments were missing or altered in size. In most ESY clones, the *Alu* pattern appeared to be intact and without significant deletion, rearrangement or segmental amplification.

Integration of YAC vector sequences was investigated with vector arm-specific probes. A 4.5 kb *Hin*dIII fragment, detected by the right arm probe in yHPRT, was observed in 10 of 20 ESY clones (Fig. 1*b*). This vector arm was lost in eight ESY clones (for example ESY 3-1, 3-6, Fig. 1*b*) and rearranged in two (for example ESY 8-6, Fig. 1*b*). The left arm probe detected the 3 kb and 4.1 kb *Hin*dIII yHPRT fragments in 18 of 20 clones (Fig. 1*c*). In total, 8 of the 20 clones (such as ESY 5-2, 8-7, 7-3, Fig. 1*a–c*) contained complete *Alu* profiles and both intact YAC vector arms.

The structural integrity of yHPRT in ESY clones 5-2 and 8-7 was further evaluated by pulsed-field gel electrophoresis. In yeast carrying yHPRT, five *Sfi*I fragments of the following rough sizes were defined by different probes: 315 kb (*Alu*, left arm),

EXHIBIT C

Molecular Membrane Biology, 1999, 16, 129–140

Lipid-based systems for the intracellular delivery of genetic drugs

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Summary

Currently available delivery systems for genetic drugs have limited utility for systemic applications. Cationic liposome/plasmid DNA or oligonucleotide complexes are rapidly cleared from circulation, and the highest levels of activity are observed in 'first pass' organs, such as the lungs, spleen and liver. Engineered viruses can generate an immune response, which compromises transfection resulting from subsequent injections and lack target specificity. A carrier, which can accumulate at sites of diseases such as infections, inflammations and tumours, has to be a small, neutral and highly serum-stable particle, which is not readily recognized by the fixed and free macrophages of the reticuloendothelial system (RES). This review summarizes lipid-based technologies for the delivery of nucleic acid-based drugs and introduces a new class of carrier systems, which solve, at least in part, the conflicting demands of circulation longevity and intracellular delivery. Plasmid DNA and oligonucleotides are entrapped into lipid particles that contain small amounts of a positively charged lipid and are stabilized by the presence of a polyethylene glycol (PEG) coating. These carriers protect nucleic acid-based drugs from degradation by nucleases, are on average 70 nm in diameter, achieve long circulation lifetimes and are capable of transfecting cells.

Keywords: gene therapy, plasmid DNA, antisense oligonucleotides, cationic liposomes, DNA encapsulation.

Abbreviations: BamHI, restriction enzyme; CL, cardiolipin; chol, cholesterol; DMRIE, 1,2-dimyristyloxy-3-(N,N-dimethyl-N-hydroxyethylammonium) propane; DODAC, N,N-dioleyl-N,N-dimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; EPC, egg phosphatidylcholine; GFP, green fluorescent protein; HII phase, inverted hexagonal phase; LUV, large unilamellar vesicle; pCMV-CAT and pCMV-βgal, plasmids containing the cytomegalovirus (CMV) promoter and coding for the marker enzymes chloramphenicol acetyltransferase (CAT) and β-galactosidase (βgal); pDNA, plasmid DNA; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PEG-CerC_8, C_{14} and C_{20}, polyethyleneglycol-modified ceramides with variable fatty acid chain lengths; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PS, phosphatidylserine; PS-oligos, phosphorothioate oligonucleotides; Rh-dextran, rhodamine-labelled dextran; SPLP, stabilized plasmid-lipid particles.

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Introduction

Genetic drugs, such as antisense oligonucleotides, and plasmids containing therapeutic genes, have considerable potential for treatment of human diseases such as cancer, infections and genetic disorders. Antisense oligonucleotides are used to decrease expression of disease-causing genes, whereas plasmids delivered to cells can cause expression of therapeutic proteins (Crooke and Bennett 1996, Akhtar and Agrawal 1997). However, rapid breakdown and clearance from the blood compromise the effectiveness of these molecules for systemic treatment of disease following intravenous injection. In addition, antisense oligonucleotides and plasmid DNA are large molecules that do not readily penetrate target cell membranes to reach their sites of action inside the cell. As a result, the development of appropriate delivery systems is critical to the clinical success of nucleic acids as pharmaceuticals. Currently, the favoured delivery systems for gene transfer are genetically engineered viruses including retroviruses, adenoviruses, adeno-associated virus (AAV) and Herpes virus (Miller and Vile 1995, Vile *et al.* 1996, Friedmann 1997, Robbins *et al.* 1998). Engineered viruses are efficient for inserting foreign genes into cells. Disadvantages of viral vectors include that they can generate an immune response, which compromises transfection resulting from subsequent injections; and that they may become pathogenic (Yei *et al.* 1994, Hope *et al.* 1998). Viral gene delivery systems are also rapidly cleared from the circulation, limiting potential transfection sites to first pass organs such as the lungs, liver and spleen. As a result of these and other limitations, there has been substantial effort focused on constructing non-viral vectors, particularly on the use of cationic lipids.

This review summarizes lipid-based technologies for the delivery of nucleic acid-based drugs. General concepts are explained first, followed by a description of the steps involved in cationic liposome-mediated gene transfer into cells. Subsequently, the role of dioleoylphosphatidylethanolamine (DOPE) and cationic lipids for the delivery of genetic drugs is described in the broader context of membrane fusion and/or destabilization (disruption). Finally, the approach taken for the systemic delivery of genetic drugs is outlined.

General concepts

Plasmid DNA and oligonucleotides

Plasmids for gene transfer consist of a bacterial plasmid backbone containing a gene encoding either a reporter protein, which allows easy quantitation of gene expression, or a therapeutic protein. The most commonly used reporters are the genes coding for luciferase, green fluorescent protein (GFP) and β-galactosidase (β-gal). Usually, the pDNA vector contains additional genetic sequences such as strong viral promoters/enhancers for efficient gene expression, select-

Molecular Membrane Biology ISSN 0968-7688 print/ISSN 1464-5203 online
http://www.tandf.co.uk/JNLS/mbc.htm
http://www.taylorandfrancis.com/JNLS/mbc.htm

able markers (antibiotic or drug resistance), transcript stabilizers, and targeting elements. One of the problems associated with DNA is the susceptibility of the phosphodiester linkage to degradation by nucleases present in serum or the intracellular environment. Unprotected DNA is degraded within minutes. Its plasma half-life after intravenous injection into mice is about 5–10 min (Kawabata *et al.* 1995).

Antisense oligonucleotide technology involves much shorter sequences of nucleic acids than the typical plasmid designed for gene therapy, and as a result has more in common with conventional drug treatment. Antisense oligonucleotides consist of short synthetic single-stranded sequences of DNA, which can bind to complementary sequences in DNA or mRNA, thereby preventing transcription and translation, respectively (Helene and Toulme 1990). In contrast to plasmid DNA oligonucleotides can be chemically modified to become more resistant against degradation by nucleases present in serum and inside cells. For example, if one of the non-esterified oxygen atoms of the natural phosphodiester backbone is replaced with sulphur, the sequence is protected from intracellular nucleases for 24–48 h, compared to an intracellular half-life for the phosphodiester of only minutes (Hoke *et al.* 1991, Fisher *et al.* 1993, Crooke 1998, Hope *et al.* 1998). The serum half-life increases by a factor of 30 to about 9 h (Campbell *et al.* 1990; Akhtar *et al.* 1991; Gilar *et al.* 1997). The resulting phosphorothioate oligodeoxynucleotides are the most frequently used class of chemically modified oligonucleotides. Table 1 summarizes physicochemical properties of a typical plasmid DNA and of oligonucleotides and compares these parameters with those of 100 nm LUVs. The dimensions of plasmid DNA and liposomes are shown in figure 1. The sizes (longest dimension) and structures of a 4.4 kbp plasmid were obtained from electron micrographs published by Lewis *et al.* (1985).

Cationic liposomes

Cationic liposomes are the most widely and successfully used lipid-based vectors for gene transfer (Felgner *et al.* 1987, Gao and Huang 1995, Ledley 1995, Felgner 1997, Chonn and Cullis 1998). In addition, they have also been used for the delivery of RNA, antisense oligonucleotides and proteins (Malone *et al.* 1989, Debs *et al.* 1990, Bennett *et al.* 1992, Walker *et al.* 1992, Dwarki *et al.* 1993, Sells *et al.* 1995). The importance of cationic liposomes as gene carriers is reflected in the wide variety of commercially available cationic liposome formulations (see table II in Sorgi and Huang 1997). The vast majority of these formulations consists of a cationic lipid mixed with DOPE at a 1:1 molar ratio. The charge of the complexes is slightly positive to allow for interaction with negatively charged cell surfaces, thus increasing cellular uptake. The transfection efficiency of any given formulation is highly dependent on the cell line, type of cationic lipid (liposome formulation), and ratio of DNA to liposomes used (Felgner *et al.* 1987, Jamagin *et al.* 1992, Stewart *et al.* 1992, Mok and Cullis 1997). The preparation procedure is simple. The cationic liposomes, usually vesicles with diameters $\leqslant$100 nm, are mixed with DNA in a dilute

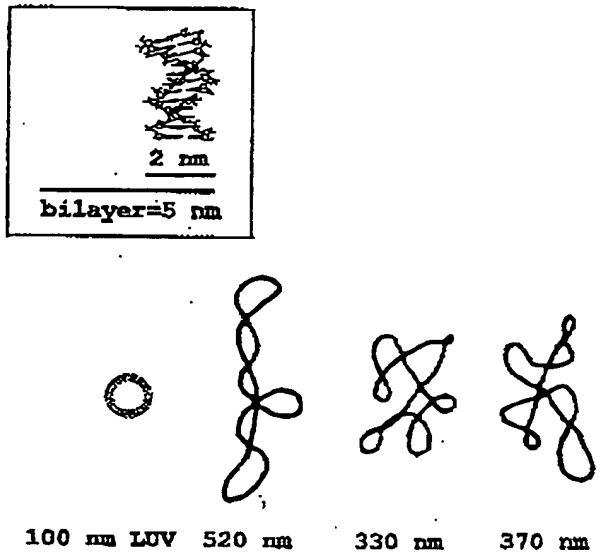

Figure 1. Comparison of the dimensions of a 100 nm LUV and a 4.4 kbp plasmid. Three different structures of the same 4.4 kpb plasmid are shown, together with their longest dimension. The bilayer thickness and DNA cross-section are not plotted according to their relative dimensions. They are shown on a correct scale in the inset. The plasmid structures were reproduced with permission from electron micrographs published by Lewis *et al.* (1985).

Table 1. Physico-chemical properties of plasmid DNA, oligonucleotides and liposomes. These parameters were obtained under the following conditions and assumptions: Plasmid DNA was modelled as a rodlike molecule with a circular cross-section of 2 nm. The contour (or extended) length of the plasmid was calculated using 0.34 nm/bp, and for the MW, an average MW of 650/bp was assumed. The MW of a 100 nm large unilamellar vesicle (LUV) was determined using 0.6 nm^2/lipid molecule for the lipid headgroup area and MWs between 630–760/lipid molecule, where 630 corresponds to the average molecular weight of a 1:1 DOPE/DODAC mixture and 670 is the MW of POPC. V_{in} is the internal aqueous volume of a 100 nm LUV, V_{mem} the volume of the lipid bilayer, O_{out} the outer surface area and O_{in} the inner surface area.

Physico-chemical property	Plasmid DNA pCMV-CAT	Typical oligo-nucleotide	100 nm LUV
Number of bases	6980	15–25	94700*
MW	2.9×10^6	$4.5–8 \times 10^3$	$60–70 \times 10^6$
Contour length (nm)	1500	<10	100†
Volume (nm^3)	4.7×10^3	–	$V_{in}=3.8 \times 10^5$ $V_{mem}=1.4 \times 10^5$
Surface area (nm^2)	9.4×10^3	–	$O_{out}=3.1 \times 10^4$ $O_{in}=2.6 \times 10^4$

*Number of lipid molecules in a 100 nm LUV. †LUV diameter.

solution. The complexes form spontaneously due to electrostatic charge interactions, which lead to liposome fusion and aggregation. The interaction between DNA and lipid is difficult to control, producing large complexes that have a very heterogeneous size distribution. Particle sizes range from 250 nm to >1 μm. The major parameters determining the final product are the charge ratio, ionic strength of the media and the overall concentration of the reactants. Structural features revealed by electron microscopy include liposome-coated DNA strands (beads on a string), aggregates of liposomes intercalating DNA, DNA entrapped between the lamellae of aggregated multilamellar structures and tubular structures consisting of fused liposomes around DNA (Gershon *et al.* 1993, Stemberg *et al.* 1994, Gustafsson *et al.* 1995, Mok and Cullis 1997).

Endocytosis

The primary route of internalization of liposomes by cells is the endocytic pathway via clathrin-coated pits (Straubinger *et al.* 1983, 1990, Daleke *et al.* 1990, Lee *et al.* 1992, Friend *et al.* 1996). Along this route, liposomes encounter compartments of progressively more acidic pH and are degraded together with their contents once they reach the lysosomes (Dijkstra *et al.* 1984, 1985, Yoshimura *et al.* 1995, Scherphof and Kamps 1998). Endocytosis is also the main mechanism by which DNA, as well as oligonucleotide-cationic liposome complexes, are taken up into the cell (Zhou and Huang 1994, Friend *et al.* 1996, Zelphati and Szoka 1996a,b). A main barrier in lipid-based drug delivery is the escape of hydrolytically sensitive material from degradation in lysosomes. The intracellular processing of liposomes and their contents is not well understood (Straubinger 1993). For example, how does the timing of release of liposomally entrapped material into endocytic compartments and its nature affect its intracellular distribution? Are there transport mechanisms other than these responsible for the rapid export of fluorescent dyes and drugs from cells (di Virgilio *et al.* 1989, Daleke *et al.* 1990, Cao *et al.* 1992, Steinberg 1994)? For example, Arabinoside C, entrapped in liposomes, is actively transported into the cytosol. Maximum cytotoxicity is attained if it is released from the liposomes into early endosomes (Brown and Silvius 1990). Furthermore, the differential effect of chloroquine with different cytofection formulations suggests that transfer to different endocytic compartments (early endosomes versus late endosomes or lysosomes) is necessary for transfection activity (Felgner *et al.* 1994).

All eukaryotic cells exhibit one or more forms of endocytosis (Mellman 1996, Robinson *et al.* 1996, Mukherjee *et al.* 1997). The best-characterized mechanism is receptor-mediated endocytosis via clathrin-coated vesicles. Clathrin-coated vesicles are also involved in liposome endocytosis (Chin *et al.* 1989). The clathrin-coated pit pathway is described below. The overall organization of the endocytic pathway is shown in figure 2. Extracellular macromolecules (ligands) bind to complementary cell-surface receptors and enter the cell together with solutes in clathrin coated vesicles (CCV) that pinch off from the cell surface. Their contents are delivered to early endosomes (EE) spread throughout the peripheral cytoplasm, where ligands and receptors are sorted to a variety of destinations. Their internal pH is only slightly acidic, ranging from 6.3–6.8. From here, endocytosed material is either directly sorted back to the plasma membrane, or may also pass through a separate, highly tubulated recycling compartment (RE), which in many cell types is located in the perinuclear region. Transit through EEs is very rapid (2–3 min) but takes longer through perinuclear REs. The transfer of material from EEs to late endosomes (LEs) involves carrier vesicles (CV) originating from EEs, which migrate on microtubules (MT) to the perinuclear region where they fuse with LEs. Ligands accumulate in LEs with a halftime of 10–25 min and encounter a pH of 5–5.5 (Schmid *et al.* 1988). The relationship between LEs and lysosomes is dynamic and not easily defined. Lysosomes contain a wide variety of hydrolytic enzymes and are the principal sites of intracellular digestion. It takes about 35 min for ligands to reach the lysosomes (pH ≤ 5) (Schmid *et al.* 1988). Endosomes communicate also with the biosynthetic pathway by vesicular transport. Newly synthesized lysosomal enzymes are delivered from the ER via the

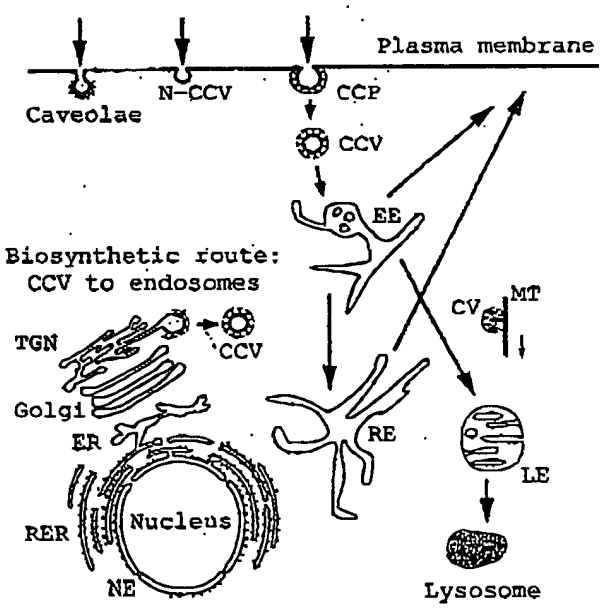

Figure 2. Organization of the endocytic pathway. Cells constantly take up material such as essential nutrients, chemical signals and also pathogens, from the extracellular medium by different forms of endocytosis. The best-characterized mechanism is the entry through clathrin-coated vesicles (CCV). However, other, non-clathrin-mediated mechanisms occur in parallel. CCV deliver their cargo to early endosomes. From there, part of the material is either directly recycled back to the plasma membrane or with a delay via recycling endosomes (RE) located in the peri-nuclear region. Material which is destined for degradation is further transported by carrier vesicles (CV) along microtubule tracks (MT) to late endosomes (LE) and finally to lysosomes, the principal sites of intracellular digestion. The endocytic pathway merges with the biosynthetic (secretory) pathway. CCV deliver digestive enzymes synthesized at the endoplasmic reticulum (ER) to endosomes. Further abbreviations found in this figure are: TGN, trans-Golgi-network; NE, nuclear envelope; N-CCV, non-clathrin coated vesicles and CCP, clathrin coated pits. This figure was adapted from Robinson *et al.* (1996).

Golgi apparatus and the trans-Golgi-network (TGN) to endosomes and then routed towards lysosomes (Kornfeld and Mellman 1989). Cells such as macrophages and fibroblasts internalize their entire plasma membrane surface area twice every hour. The extracellular fluid thereby taken up corresponds to 25% of their internal volume. Each LDL receptor makes one round trip every 12 min, or 150 round trips in its 30 h lifespan (Brown *et al.* 1983).

Cationic liposome-mediated delivery of genetic drugs into cells

Plasmid DNA

Cationic liposomes are the most intensively investigated non-viral gene transfer vectors (Felgner *et al.* 1987, Gao and Huang 1995, Felgner 1997). Many steps of the cationic liposome-mediated gene transfer process have been identified. Factors which influence gene expression are: (1) uptake of the DNA-liposome complexes into the cell, (2) release from endo/lysosomal compartments, (3) dissociation of the DNA from its interaction with the cationic lipid, (4) DNA transport across the nuclear envelope, and (5) gene expression (transcription and translation).

The main route of entry of cationic liposome/DNA complexes into cells is by endocytosis (Zhou and Huang 1994, Friend *et al.* 1996). Uptake is facilitated by a net positive charge on the complexes. In a typical transfection experiment using DMRIE/DOPE, COS-1 cells take up approximately 3×10^5 plasmids/cell, while only 50% of the cells expressed the transgene (Zabner *et al.* 1995). Once the complexes are taken up into the cell they are transported to the lysosomes for degradation. In order for gene expression to occur, DNA has to escape the endocytic pathway and redistribute into the nucleus in intact form. Endosomal membranes appear to be extensively destabilized upon interaction with cationic liposome/DNA complexes. Transmission electron microscopy pictures, for example, show disrupted endosomal membranes (Zhou and Huang 1994, El Ouahabi *et al.* 1997). The authors' own experiments demonstrate release of rhodamine-labelled dextran (Rh-dextran, MW 10000) into the cytosol upon co-incubation with DOPE/DODAC liposome/DNA complexes (figure 3, bottom). At the same time, extensive mixing of the lipids in the complex with cellular membranes takes place (Wrobel and Collins 1995, Stegmann and Legendre 1997). It was further proposed that the destabilization of the endosomal membrane by the internalized complexes induces flip-flop of anionic lipids from the cytoplasmic-facing monolayer. Formation of a charge-neutral ion pair would then result in displacement of the DNA from the cationic lipid and release of the DNA into the cytoplasm (Xu and Szoka 1996). Most of the DNA, however, remains localized in endocytic compartments and is degraded. Only a small fraction escapes into the cytoplasm in intact form.

A further barrier in cationic liposome-mediated gene transfer is the translocation of the plasmid across the nuclear envelope (Zabner *et al.* 1995, Wrobel and Collins 1995, Zabner 1997, Pollard *et al.* 1998). It was found that microinjection of plasmid DNA into the cytoplasm of cells does not support substantial levels of gene expression. Direct introduction of DNA into the nucleus is required for high levels of expression to occur (Capecchi 1980, Mirzayans *et al.* 1992). Gene expression following cytoplasmic microinjection of naked DNA reaches a maximum 24 h post-injection (Pollard

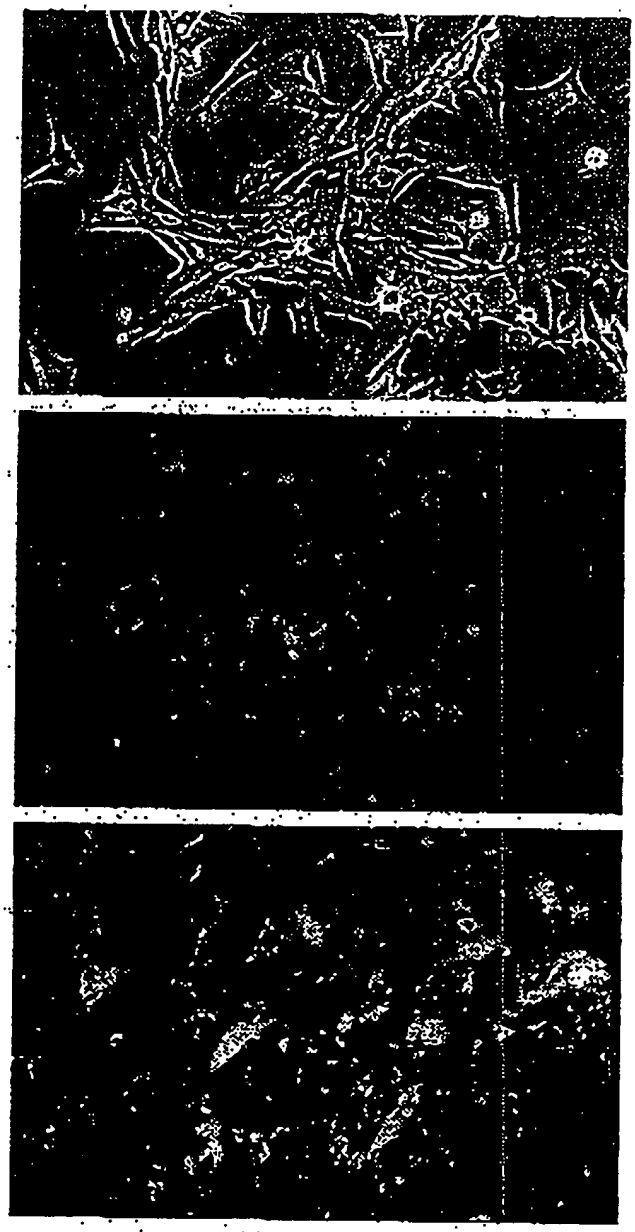

Figure 3. Membrane-destabilizing effect of DOPE, demonstrated by the cationic liposome mediated release of fluorescently labelled dextran into the cytosol. BHK cells were co-incubated with DOPE/DODAC or DOPC/DODAC cationic liposome/DNA complexes (+charge ratio 1.5 and 0.5 μg pCMV-βgal plasmid) and 1 mg/ml rhodamine-labelled dextran (Rh-dextran, MW 10000). After 24 h, release of Rh-dextran into the cytosol can only be observed for PE-containing complexes (bottom panel) but not for PC-containing complexes (middle panel). The top panel shows the corresponding phase contrast picture of the cells incubated with the PC complexes. Incubation with Rh-dextran alone resulted in punctate fluorescence (not shown).

et al. 1998). Its efficacy is lower by a factor of 1000 compared to direct nuclear injection (10000 copies of naked plasmid injected into the cytoplasm of COS-7 cells gave the same level of transfection (13%) as 10 copies injected into the nucleus) (Zabner *et al.* 1995, Pollard *et al.* 1998). Also, transgene expression is greater with plasmids (naked as well as complexed with cationic liposomes) that do not require nuclear transcription (e.g. T7 promoter-driven gene expression system together with T7 RNA polymerase) (Rose *et al.* 1991, Gao and Huang 1993, Zabner *et al.* 1995). Mitotic cells show increased transfectability (Zabner *et al.* 1995, Wilke *et al.* 1996, Mortimer *et al.* 1998, Zelphati *et al.* 1998). This limits the number of cells amenable to transfection to the fraction of cells undergoing cell division. These results demonstrate that trafficking of DNA from the cytoplasm to the nucleus is very inefficient and is facilitated by the disassembly of the nuclear envelope during cell division. The inefficient transfer of plasmid DNA to the nucleus increases its exposure time to cellular nucleases. Naked plasmid DNA has a half-life of about 2 h in the cell cytoplasm (75% degraded after 4 h) (Mirzayans *et al.* 1992, Lechardeur *et al.* 1999). Increased transgene expression following cytoplasmic microinjection of DNA has been observed when DNA was complexed with polyethyleneimine, but not for DNA complexed with cationic liposomes (Page *et al.* 1995, Zabner *et al.* 1995, Pollard *et al.* 1998). Further, it was shown that the microinjection of DNA/liposome complexes into the nucleus of oocytes resulted in no detectable level of expression when compared to free DNA, indicating that the lipid coating of the DNA inhibits transcription and has to be removed before the DNA enters the nucleus (Zabner *et al.* 1995).

Oligonucleotides

Phosphodiester oligonucleotides are rapidly degraded in biological fluids. Therefore, most antisense studies and clinical trials have involved chemically modified, more resistant oligonucleotides, in particular phosphorothioate oligonucleotides (Akhtar and Agrawal 1997, Crooke 1997, Stein 1998). A potential problem associated with free oligonucleotides is their poor uptake by most cell lines *in vitro* and export from cells (Crooke 1991, Marti *et al.* 1992, Stein and Cheng 1993, Tonkinson and Stein 1994). In addition, sequestration of oligonucleotides into endosomal compartments decreases their intracellular availability. Cationic lipids have been shown to enhance uptake of oligonucleotides into cells (Bennett *et al.* 1992).

When antisense oligonucleotides are given to cells in the form of 'complexes' with cationic liposomes, endocytosis and rapid movement to the nucleus occur (Zelphati and Szoka 1996a,b). This is shown in figure 4. The oligonucleotide/cationic lipid complex dissociates, with the oligodeoxynucleotide entering the nucleus and the cationic lipid as well as DOPE remaining in the endosomal compartments (Marcusson *et al.* 1998). Rapid nuclear accumulation of fluorescently labelled oligonucleotides can also be observed upon microinjection into the cell cytoplasm (Chin *et al.* 1990, Leonetti *et al.* 1991, Fisher *et al.* 1993, Sixou *et al.* 1994). Nuclear transport occurs mainly by diffusion and does not appear to be severely affected by depletion of the intracellular ATP pool or excess unlabelled oligomer (Chin *et al.* 1990, Leonetti *et al.* 1991). Binding to nuclear structures is responsible for retention (accumulation) in the nucleus (diffuse and bind model). PS-oligonucleotides were found to be unevenly distributed across the nucleus, concentrated in small foci (Lorenz *et al.* 1998). In contrast to plasmid DNA, the smaller size of the oligonucleotides allows them to translocate across the nuclear envelope through the nuclear pores. These pores act like a size exclusion sieve, with

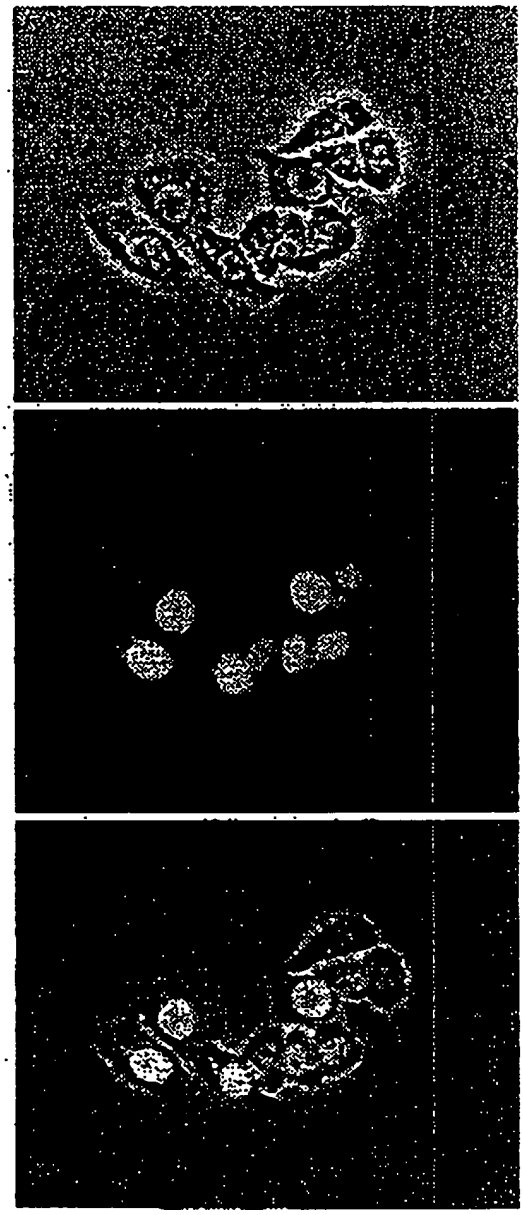

Figure 4. Rapid nuclear accumulation of fluorescently labelled phosphorothioate oligonucleotides (PS-oligos) mediated by DOPE/DODAC cationic liposomes. SK-BR-3 cells were incubated for 4 h with 3 μg of FITC-labelled PS-oligos complexed with DODAC/DOPE (1 : 1) at a positive-to-negative charge ratio of 1.5. Cells were washed and observed by phase contrast (upper panel) or fluorescence microscopy (middle panel). The merged photographs are presented in the bottom panel.

macromolecules as large as 60 kDa being able to pass through (Peters 1986, Dingwall 1991).

Membrane fusion versus membrane destabilization

DOPE and its ability to induce lipid mixing are automatically associated with membrane fusion. Membrane fusion in biological systems is usually thought to be a non-leaky process. However, there is evidence that this is not always the case. For example, influenza virus-mediated fusion appears to be a highly leaky process with extensive disruption of the lipid membrane at the fusion site (Shangguan *et al.* 1996). The classical picture of a non-leaky fusion event certainly does not apply to the cationic liposome-mediated gene transfer process, where the endosomal membrane is significantly disrupted upon interaction with the cationic liposome/DNA complexes (see figure 3). In both cases, extensive lipid mixing was observed. Lipid mixing, often equated with fusion, is in these cases only an expression of extensive membrane destabilization. Under these circumstances the definition of fusion has to be broadened to allow for massive membrane destabilization (disruption).

Membrane fusion

Membrane fusion is a fundamental process in many cellular functions, including endocytosis, exocytosis/secretion, and cell division and the mechanism by which viruses enter cells (White 1992). Membrane fusion in biological systems is an extremely fast and local event that involves only a very small surface area of the interacting membrane (Burger 1997). It is essentially non-leaky and involves proteins that bring the fusing membranes into close proximity and promote fusion through membrane destabilization. Proteins determine when and where membrane fusion occurs. The central event in membrane fusion, however, is the merger of two membranes. This requires a transient reorganization of membrane lipids (disruption of the lipid bilayer structure). During this reorganization intermediate structures are formed (Cullis and Hope 1978, Siegel 1986, Siegel and Epand 1997).

DOPE and cationic lipids as membrane-destabilizers

Unsaturated phosphatidylethanolamines (PEs) are common membrane phospholipids which, in isolation, spontaneously adopt the inverted hexagonal phase (H_{II} phase). They have been proposed to play a key role in biomembrane fusion facilitating the formation of highly curved semifusion intermediates and stimulating membrane fusion (Hope and Cullis 1981, de Kruijff *et al.* 1985, Ellens *et al.* 1986, Bailey and Cullis 1997a,b). Unsaturated PEs can adopt a bilayer structure in the presence of stabilizing lipids such as PCs, detergents and PEG-lipid constructs (Madden and Cullis 1982, Seddon 1990, Litzinger and Huang 1992, Holland *et al.* 1996a). Loss of the stabilizing function results in H_{II} phase formation and is, for liposomes, accompanied by leakage of contents. Formation of non-bilayer structures also results in extensive lipid mixing.

Most of the lipofectin formulations presently studied require PE for optimal activity. The dependence of efficient transfection on the presence of unsaturated PE as a helper lipid has been observed for a variety of cationic lipids in many different cell lines *in vitro* (Felgner *et al.* 1994, Gao and Huang 1995). The strong DOPE dependence is related to the membrane-destabilizing activity of DOPE (Farhood *et al.* 1995). This is demonstrated in figure 3. Cytoplasmic release of Rh-dextran requires DOPE (figure 3, bottom) and does not occur to a detectable level in the presence of dioleoylphosphatidylcholine (DOPC) (figure 3, middle). Successive methylation of the PE headgroup and increasing acyl chain saturation reduces transfection efficiency (Felgner *et al.* 1994). Cationic liposome formulations containing DOPE were also more active in inducing lipid mixing than DOPC formulations in model membrane studies as well as in *in vitro* experiments (Stamatatos *et al.* 1988, Düzgüneş *et al.* 1989, Leventis and Silvius 1990, Wrobel and Collins 1995, Bailey and Cullis 1997a,b). Cationic liposomes are capable of inducing haemolysis of erythrocytes and of destabilizing isolated lysosomes (Yoshihara and Nakae 1986, Wattiaux *et al.* 1997, Mui, unpublished observations). Lipid mixing between cationic liposomes and anionic liposomes, as well as the haemolytic activity of cationic liposomes, is strongly reduced by DNA and in the presence of serum (Leventis and Silvius 1990, van der Woude *et al.* 1995, Bailey and Cullis 1997, Mui, unpublished observations). Free DOPE-containing cationic liposomes (helper liposomes) can significantly enhance the transfection activity of the complexes (Farhood *et al.* 1995, Li *et al.* 1998).

The cationic lipid component mediates association of the liposomes with the nucleic acid polyanions through electrostatic interactions, thus allowing the complexes to be formed. The interaction with the cationic liposomes results in a partial protection of these molecules against hydrolytic enzymes. A net positive charge of the complexes facilitates uptake into cells by allowing efficient interaction with the negatively charged cell surface. The cationic lipid-mediated association with cell membranes may initiate membrane fusion and disruption. It was shown that anionic liposomes can dissociate DNA from its interaction with cationic liposomes (Xu and Szoka 1996). Destabilization of the endosomal membrane by the complexes with concomitant displacement of the cationic lipid by ion pair formation with anionic cellular lipids may be responsible for the release of nucleic acid-based drugs from endocytic compartments into the cytosol (Zelphati and Szoka 1996). In general, the transfection activity of cationic lipids decreases with increasing alkyl chain length and saturation. It was suggested that the higher intermembrane lipid transfer rates and faster rates of intermembrane lipid mixing of cationic lipids with shorter alkyl chain lengths could contribute to the destabilization of the endosome (Silvius and Leventis 1993, Felgner *et al.* 1994).

Towards systemic delivery of genetic drugs

Currently available delivery systems for genetic drugs have limited utility for systemic applications. The large size and positively charged character of cationic liposome/plasmid DNA or oligonucleotide complexes result in rapid clearance from circulation, and the highest levels of activity are

observed in 'first pass' organs, particularly the liver and lungs (Mahato *et al.* 1995, Huang and Li 1997, Chonn and Cullis 1998). Oligonucleotides show efficacy in free form *in vivo*, however, they also lack site-selective accumulation (Agrawal 1996, Akhtar and Agrawal 1997). The need for a delivery system for treatment of systemic disease is obvious. For example, for cancer gene and oligonucleotide therapy there is a vital need to access metastatic disease sites as well as primary tumours. Similar considerations apply to other systemic disorders, such as inflammatory diseases. The design features for lipid-based delivery systems that preferentially access such disease sites are increasingly clear from studies on liposomal systems containing conventional drugs, where it has been shown that small (diameter < 100 nm), long-circulating vesicles preferentially accumulate at sites of infection, inflammation and tumours (Gabizon and Papahadjopoulos 1988, Chonn and Cullis 1995). Thus, the carrier containing genetic drugs should be a small, neutral and highly serum-stable particle, which is not readily recognized by the fixed and free macrophages of the reticuloendothelial system (RES). However, in order to maximize potency after arrival at a disease site, the particle should interact readily with cells and have the ability to destabilize cell membranes to promote intracellular delivery of the active agent. The approach taken to solve these problems is described below.

Encapsulation of plasmid DNA in stabilized plasmid-lipid particles

Plasmid DNA has been encapsulated by a variety of methods (see table 2). None of these procedures yields small, serum-stable particles at high plasmid concentrations and plasmid-to-lipid ratios in combination with high plasmid encapsulation efficiencies. In particular, passive encapsulation of plasmid DNA in liposomes is very inefficient due to the large size of these molecules. Efficient entrapment requires the interaction of the lipid components with the DNA with a concomitant reduction in DNA size. Cationic lipids fulfil these requirements (Düzgüneş and Felgner 1993, Bloomfield 1996, Lasic 1997). Neutralization of the negative phosphate charges through association with cationic lipids decreases repulsion between DNA segments and allows bending of DNA and a reduction in size. However, the strong electrostatic interaction between DNA and cationic lipid is difficult to control. Membrane fusion events and aggregation usually result in the production of large and heterogeneous aggregates. Therefore, regulatory components, which allow the control of these processes, are required. For example, macromolecules can be sterically excluded from the liposomal surface by incorporation of PEG-lipid conjugates into the liposomal membrane. This forms the basis of sterically stabilized liposomes, where reduction of the level of plasma

Table 2. Procedures for encapsulating pDNA in lipid-based systems. The following table was adapted from Wheeler *et al.* (1999). ND stands for not determined; DLS for dynamic light scattering and EM for electron microscopy. *Some values were calculated based on presented data.

Procedure	Lipid composition	Trapping efficiency*, size of DNA	DNA-to-lipid ratio*	Diameter	References
Reverse phase evaporation	PS or PS:Chol (50:50)	30–50%, SV40 DNA	<4.2 μg/μmol	400 nm	Fraley *et al.* 1980
Reverse phase evaporation	PC:PS:Chol (40:10:50)	13–16%, 11.9 kbp	0.23 μg/μmol	100 nm to 1 μm	Soriano *et al.* 1983
Reverse phase evaporation	PC:PS:Chol (50:10:40)	10%, 8.3 and 14.2 kbp	0.97 μg/μmol	ND	Nakanishi *et al.* 1985
Reverse phase evaporation	EPC:PS:Chol (40:10:50)	12%,3.9 kbp	0.38 μg/μmol	400 nm	Cudd and Nicolau 1985
Ether injection	EPC:EPG (91:9)	2–6%, 3.9 kbp	<1 μg/μmol	0.1–1.5 μm	Fraley *et al.* 1979
Ether injection	PC:PS or PG:Chol (40:10:50)	15%, 3.9 kbp	15 μg/μmol	ND	Nicolau and Rottem 1982
Detergent dialysis	EPC:Chol:stearylamine (43.5:43.5:13)	11% , sonicated genomic DNA	0.26 μg/μmol	50 nm	Stavridis *et al.* 1986
Detergent dialysis, extrusion	DOPC or DOPE:Chol: oleic acid (40:40:20)	14–17%, 4.6 kbp	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)	Wang and Huang 1987
Lipid hydration	EPC:Chol (65:35) or EPC	ND, 3.9 and 13 kbp	ND	0.5–7.5 μm	Lurquin 1979
Dehydration-rehydration, extrusion (400 or 200 nm filters)	Chol:EPC:PS (50:40:10)	ND.	0.83–1.97 μg/μmol	54.6 nm and 142.5 nm	Alino *et al.* 1993
Dehydration-rehydration	EPC	35–40%, 2.96, 7.25 kbp	2.65–3.0 μg/μmol	1–2 μm	Baru *et al.* 1995
Sonication (in the presence of lysozyme)	Asolectin (soybean phospholipids)	50%, 1 kbp linear DNA	0.08 μg/μmol	100–200 nm	Jay and Gilbert 1987
Sonication	EPC:Chol:lysine-DPPE (55:30:15)	60–95% , 6.3 kbp ssDNA	13 μg/μmol ssDNA	100–150 nm	Puyal *et al.* 1995
Spermidine-condensed DNA, sonication, extrusion	EPC:Chol:PS or EPA or CL (40:50:10)	46–52%, 4.3 and 7.2 kbp	2.53–2.87 μg/μmol	400–500 nm	Ibanez *et al.* 1997
Ca^{2+}-EDTA entrapment of DNA-protein complexes	PS:Chol (50:50)	52–59%, 42.1 kbp bacteriophage	22 μg/μmol	ND	Szoka and Duda 1989
Freeze-thaw, extrusion	POPC:DDAB (99:1)	17–50%, 3.4 kbp linear	ND	80–120 nm	Monnard *et al.* 1997
Stabilized plasmid-lipid particles (SPLP)	DOPE:PEG-Cer: DODAC (84:10:6)	60–70%, 4.4–10 kbp plasmid	62.5 μg/mmol	65–75 nm (DLS, EM)	Wheeler *et al.* 1999

protein binding results in much longer blood circulation lifetimes. Further, PEG-lipid constructs can inhibit fusion and aggregation of charged liposomes by preventing close membrane approach (Holland *et al.* 1996b). In the following, it is demonstrated that it is possible to use PEG-lipid constructs to regulate fusion and aggregation events following the interaction of plasmid DNA with cationic lipids.

Figure 5 demonstrates that plasmid DNA can be efficiently entrapped in DOPE/DODAC/PEG-ceramide (84 : 6 : 10 mol%) systems termed 'stabilized plasmid-lipid particles' (SPLP) employing a detergent dialysis procedure (Wheeler *et al.* 1999). The trapping efficiencies are a function of the relative amounts of cationic lipid and PEG-ceramide and the ionic strength of the medium. In the absence of PEG-ceramide precipitation occurs. In these system the plasmid DNA is protected from degradation by DNase I and serum nucleases. Long circulation lifetimes can be achieved when PEG-ceramide C_{20} is used, which does not readily exchange out of the membrane. These systems thus rely on the stabilizing effects of PEG coatings and become progressively destabilized as the PEG coating dissociates from the liposomes. The rate of exchange of PEG-lipid conjugates out of the membrane depends on the acyl chain lengths and their degree of saturation (Silvius and Zuckermann 1993, Holland *et al.* 1996b). Typical exchange half-times of PEG-ceramides with acyl chain lengths from 8 to 20 carbons range from several minutes to several days (Wheeler *et al.* 1999). This allows one to adjust the rate at which the stabilizing coating dissociates from the liposomes.

The cryo-EM picture in figure 6 shows that SPLP have the morphological features of large unilamellar liposomes (LUV).

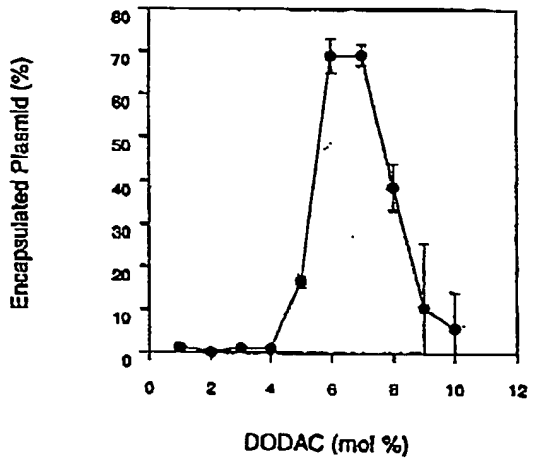

Figure 5. Effect of cationic lipid concentration on the encapsulation efficiency of plasmid DNA (pCMV-CAT) in DOPE/DODAC/PEG-ceramideC_{20} SPLP. Ten milligrams of lipid were dissolved in 200 mm octylglucoside and mixed with 50 μg plasmid DNA in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube and dialysed against HBS for 36 h at 20°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography. DNA was quantitated using either ^{3}H-labelled plasmid or the DNA intercalating fluorescent dye, PicoGreen (Molecular Probes). Lipid concentrations were determined by chromatography or with radiolabelled lipids. This figure was taken from Wheeler *et al.* (1998).

The average lipid bilayer thickness is 5.1 nm, as determined by small-angle X-ray scattering. The average diameter from dynamic light scattering measurements and freeze-fracture electron microscopy studies is approximately 70 nm. Analysis of the plasmid-to-lipid ratio reveals that each SPLP contains one plasmid (Wheeler *et al.* 1999).

Procedures have also been developed for the efficient entrapment of antisense oligonucleotides. ICAM-1 entrapped in liposomes containing a protonable cationic lipid and PEG-ceramide has been shown to elicit a strong anti-inflammatory effect in an *in vivo* ear inflammation model upon intravenous injection, where the efficacy of the liposomally entrapped ICAM-1 is much higher than for the free oligonucleotide (unpublished observations).

Circulation life-times and tumour accumulation of SPLP

Stabilized plasmid/lipid particles show long circulation lifetimes and facilitate the preferential accumulation of intact plasmid DNA in disease sites such as tumours following intraveneous injection. Figure 7a shows that 80% of the lipid with roughly the same amount of intact plasmid DNA remained in circulation after 1 h and slowly decreased to 10–20% in the course of 24 h (unpublished observations). Using a mouse tumour model and PEG-CerC_{20} 6% of the injected dose (30 μg plasmid, 2 mg lipid) was found in the tumour (figure 7b) (upublished observations).

The low levels of transfection observed with these systems appeared to be related to the low level of cellular uptake (unpublished observations). Increasing cellular uptake will be the focus of future work.

Conclusion

Delivery systems for systemic applications must have the potential to selectively deliver genetic drugs to specific target

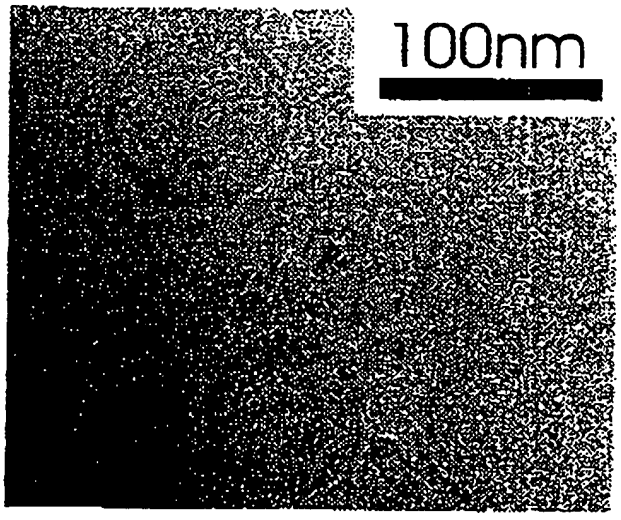

Figure 6. Cryo-electron microscopic picture of stabilized plasmid-lipid particles (SPLP). The plasmid-lipid system has the morphological features of conventional LUV. The picture was taken by Holger Stark, Imperial College, London.

sites while avoiding recognition by cells of the reticuloendothelial system. The stabilized plasmid-lipid particles introduced above solve, at least in part, the conflicting demands of circulation longevity and intracellular delivery.

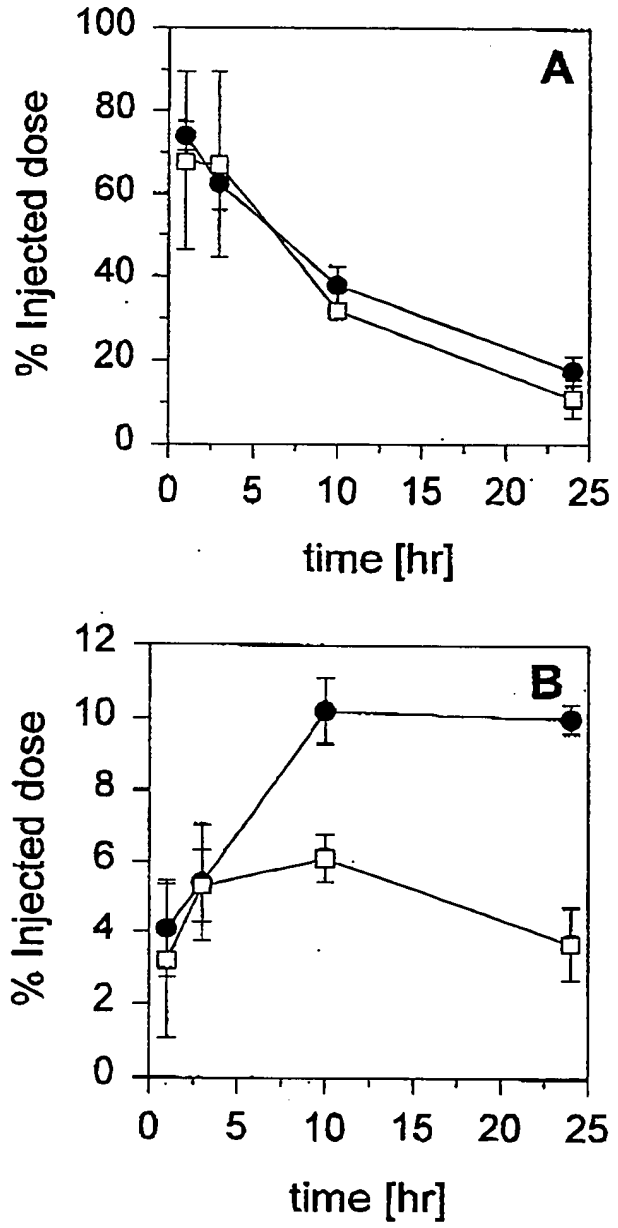

Figure 7. Tumour accumulation and plasma clearance of SPLP containing PEG-$CerC_{20}$ in BDF-1 mice bearing a Lewis lung tumour. Mice were seeded with tumour cells and, after 14 days, injected with the stabilized plasmid/lipid system at a dose of 30 μg plasmid DNA (pCMV-CAT) and 2 mg lipid. Animals were sacrificed 1, 3, 10 and 24 h post-injection. Plasma samples (a), and tumour tissue (b) were analysed for ^{3}H-labelled lipids by scintillation counting and for intact plasmid DNA by Southern blot analysis. The amount of lipid (full circles) and intact plasmid (open squares) recovered from tumour tissue and blood are given as per cent injected dose and plotted as a function of time following injection.

Future work will focus on the improvement of cellular uptake employing specific targeting ligands attached to the surface of these carriers.

Acknowledgements

We would like to thank Holger Stark from the Imperial College in London for doing the cryoelectron microscopy. This work was supported by the Medical Research Council of Canada and INEX Pharmaceuticals together with the National Science and Engineering Research Council of Canada through the Collaborative Research and Development Program.

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